ATTENUATION OF DECAPENTAPLEGIC SIGNALING VIA A LOCALIZED CHONDROITIN-SULFATED SIGNALING SINK

by

Gregory Blaine Humphreys

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STATEMENT OF DISSERTATION APPROVAL

The following faculty members served as the supervisory committee chair and members for the dissertation of Gregory Blaine Humphreys.

Dates at right indicate the members' approval of the dissertation.

Anthea Letsou, Chair	July 7, 2014 Date Approved
Carl Sennrich Thummel, Member	July 7, 2014 Date Approved
Janis Weis, Member	July 7, 2014 Date Approved
Donald McClain, Member	July 7, 2014 Date Approved
Shigeru Sakonju, Member	_____ Date Approved

The dissertation has also been approved by Lynn Jorde Chair of the Department of Human Genetics

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Glycosaminoglycans (GAGs) are long linear polysaccharides made of disaccharide repeats. The GAGs heparan and chondroitin are found ubiquitously on cell surfaces in many organisms and are involved in regulating developmental signaling, immunity, and mediating many cell-cell interactions. In *Drosophila melanogaster,* GAGs are synthesized downstream of the gene *mummy (mmy)*, which encodes the *Drosophila* UDP-N-acetylglucosamine pyrophosphorylase, the enzyme performing the final catalytic step in UDP-GlcNAc synthesis. *mmy* encodes an antagonist of Decapentaplegic (Dpp) signaling, and *mmy* mutant embryos have expanded ectopic Dpp activity in the dorsal epidermis. We confirmed through multiple tests that the *mmy*-mediated effects on Dpp signaling occur downstream of *dpp* transcription and that *mmy* activates a switch from short-range to long-range signaling in the epidermis. To identify downstream effectors of Dpp signal restriction, we screened 23 of the 25 Drosophila β-1,3-glycosyltransferases functioning downstream of Mmy. Embryos depleted of either *wanderlust* (*wand*), which encodes a putative chondroitin sulfate synthase, or *super sex combs*, which encodes an O-GlcNAc transferase, had Dpp activity expanded ectopically beyond the LE epidermis, identifying these transferases as a Dpp antagonists. We further characterized *wand* and determined that it is expressed in embryonic cardiac cells and that it antagonizes Dpp signaling in the mesoderm as well as the epidermis. Taken together, these data suggest that *mmy*, through *wand*, synthesizes a chondroitin-sulfated sink that alters the signaling range of Dpp. Future work will involve characterizing the role of other chondroitin sulfate-synthesizing genes in Drosophila signaling regulation, determining the nature of the Dpp-chondroitin interaction, exploring the role of chondroitin sulfate in imaginal disc and testes signaling, and identifying the specific GAG-modified targets that enact epidermal signal regulation. These data will provide new insights into regulation of Dpp signaling via glycosylation.

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CHAPTER 1

THE FUNCTION OF GLYCOSAMINOGLYCANS IN SIGNAL REGULATION

The role of glycosaminoglycan (GAG) in regulation of extracellular signaling has been extensively studied. GAGs are linear polysaccharides attached to extracellular proteins that are found almost ubiquitously on cell surfaces and in extracellular matrix (Abrahamsohn et al., 1975; Carey, 1997; Izumikawa et al., 2014; Woods and Couchman, 1998). Many GAGs are found enriched on the basement membranes of polarized cells, where they perform special functions (Hassell et al., 1980; Kanwar and Farquhar, 1979; Myers et al., 1996). GAGs interact with integrins, cytokines, growth factors, and other molecules to enable proper development, immunity, and homeostasis (Coombe, 2008; Frey et al., 2013; Iida et al., 1998; Jackson et al., 1997; Johnson et al., 2005; Sirko et al., 2007; Wrenshall and Platt, 1999). Three types of linear GAG modifications are observed in nature: heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS) (Bülow and Hobert, 2006).

Synthesis and subsequent modification of GAGs

Synthesis of GAGs requires a number of specific enzymes (reviewed in Bülow and Hobert, 2006). Synthesis begins with addition of the GAG-protein linker region, a tetrasaccharide that is common to all three forms of GAG. The GAG-protein linker region, which consists of GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl β1-*O*-Ser, is synthesized by stepwise addition of four sugars by the sequential action of four sugar transferases (Sugahara and Kitagawa, 2000). Xylose is attached to serine residues on the core protein by xylosyltransferase, galactose in a β1-4 linkage by galactosyltransferase I, galactose in a β1-3 linkage by galactosyltransferase II, and glucoronic acid by glucuronyltransferase I (Sugahara and Kitagawa, 2000). Polymerization of the HS and

CS chains each requires two different enzymatic activities: GlcA transferase and GalNAc transferase for HS polymerization and GlcA transferase and GlcNAc transferase for CS polymerization. These activities are contained within the same protein in the case of CS polymerization; Chondroitin sulfate synthase-1 and Chondroitin sulfate synthase-2 are dual functioning, having enzymatic domains to carry out both GlcA and GalNAc transfer (Kitagawa et al., 2001; Yada et al., 2003a). DS is not polymerized in this fashion, but is generated from mature CS polymers.

GAG chains are modified subsequent to polymerization, forming a wide diversity of sulfated disaccharides through the modification by sulfotransferases and sulfatases (Bülow and Hobert, 2006). These modifications contribute substantial complexity to GAG structure, in excess of the structural diversity of nucleotides and peptides. CS, for example, can be modified at C-2 of uronic acid residues, as well as at C-4 and C-6 of GalNAc residues; each repeating disaccharide unit may have between zero and three of these potential modifications (Figure 1.1). Organisms can vary greatly in potential GAG diversity. CS from Drosophila has a low amount of sulfation (and only occurring on C-4 of GalNAc), while C. elegans lacks sulfated chondroitin altogether and even lacks homologous enzymes to carry out the reaction (Toyoda et al., 2000). In some organisms CS can be converted to dermatan sulfate by the activity of chondroitin-glucuronate C5-epimerase (EC 5.1.3.19), though dermatan sulfate is not present in Drosophila (Maccarana et al., 2006). The presence of any iduronic acid in the GAG chain is sufficient for it to be characterized as DS, and CS must contain glucuronic acid exclusively (Béchard et al., 2001). The enzymes that perform sulfation of CS in Drosophila have not yet been identified, but BLAST analysis indicates that CG31743 has the highest homology to human Carbohydrate sulfotransferase 13, which is required to synthesize CS-A (Kang et al., 2002).

Sulfation affects proteoglycan function

The sulfation pattern of GAGs is integral to their function and activity. For instance, mutants for the Drosophila gene *sulfateless*, which is required to add sulfates to CS and HS chains, were noted to have a phenotype similar to that seen in FGF pathway mutants and in

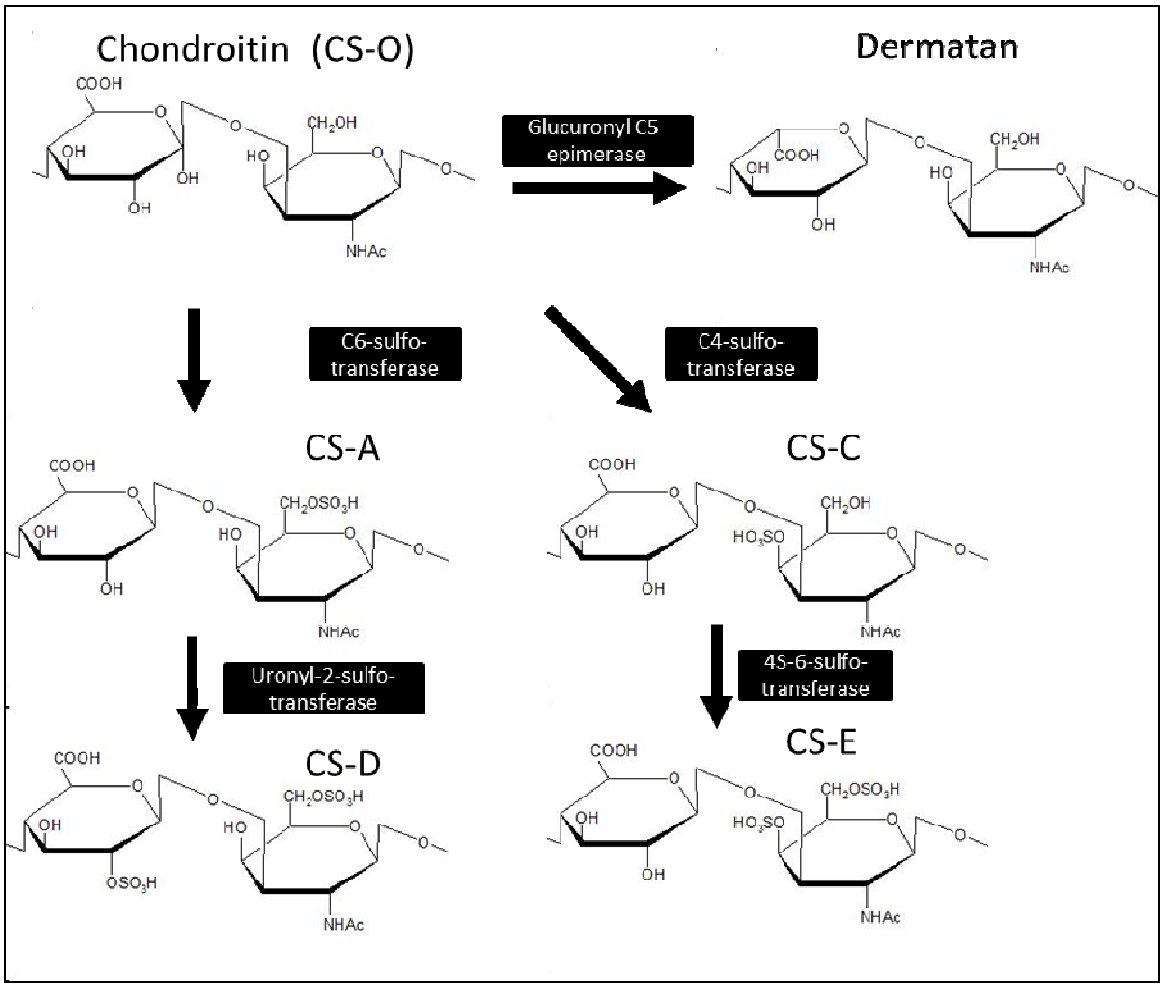

Figure 1.1. Modification of CS. Individual naked chondroitin sulfate disaccharides (CS-O) can be modified by one of three enzymes. Glucoronyl C5-epimerase converts CS-O to dermatan (Tiedemann et al., 2001); dermatan has its own subsequent modifications that are not covered here. Sulfation of CS-O by C6-sulfotransferase forms CS-A (Habuchi et al., 1993). CS-A can be further sulfated by Uronyl-2-sulfotransferase to form CS-D (Kobayashi et al., 1999). A separate modification branch is initiated by sulfation of CS-O by C4-sulfotransferase to form CS-C (Yamauchi et al., 2000). CS-C can be further sulfated by 4S-6-sulfotransferase to generate CS-E (Yamaguchi et al., 2007).

particular mutants of FGF-receptors *heartless* and *breathless* (Lin and Perrimon, 1999) .

Sulfateless is a N-deacetylase-N-sulfotransferase that modifies HS chains by removal of acetyl groups from N-acetyl-glucosamine and replacing it for sulfate (Lin and Perrimon, 1999). These results suggest that changes in sulfation pattern can promote or abolish interactions with other molecules; furthermore, desulfation might be a tool utilized by organisms to dynamically alter signal regulation by GAGs.

The unique structure of GAG allows it to interact with many molecules

After completion of polymerization and modification, the resultant GAG is one of the most negatively charged molecules in the organism. The unique structure and charge of GAGs enable them to interact with a number of growth factors, signaling molecules, enzymes, and many viral and bacterial proteins (Rostand and Esko, 1997; Salmivirta et al., 1996). Due to their ubiquity and structure, many bacteria, viruses, and parasites have developed methods to exploit GAGs to facilitate attachment, invasion, and colonization of host organisms (Rostand and Esko, 1997). Some bacteria have even developed mechanisms to release GAGs from cell surfaces in order to inactivate innate antimicrobial defenses (Schmidtchen et al., 2001). Because of the high amount of negative charge found on GAG chains, interactions with other molecules are often, but not exclusively, ionic in nature (Salmivirta et al., 1996). As GAGs carry a large negative charge, proteins that interact with them tend to have a large cluster of positively charged amino acids in the binding site (Salmivirta et al., 1996).

The roles of GAGs in developmental signaling

The function of HS in developmental signaling

The role of GAGs in extracellular signaling was first suggested by genetic screens in Drosophila. Screens for zygotic lethal genes led to the isolation of pattern formation mutants, including the signaling morphogen genes *wingless* (*wg*) and *hedgehog* (*hh*) (Perrimon et al., 1996). Wingless (Wg) and Hedgehog (Hh) exhibit a very specific ventral larval cuticle defect in which the naked cuticle is lost from the ventral cuticle, resulting in a continuous lawn of ventral denticles (Bejsovec and Wieschaus, 1993). However, these screens also uncovered many other genes involved in HS synthesis that shared loss-of-function phenotypes with *hh* and *wg* mutants. These HS synthesis genes included *sugarless, tout-velu, sulfateless, slalom,* and *dally,* which encodes an HSPG (Perrimon et al., 1996); these results suggested a role for HS in regulating extracellular developmental signals. CS synthesis genes have not been as abundantly recovered from these screens, perhaps due in part to redundancy among CS synthesizing enzymes.

The necessity for proper formation of HS chains in signaling soon became clear; clones

mutant for the *tout-velu*, *sister of tout-velu*, and *brother of tout-velu* genes, as well as *N*-acetylglucosamine transferases of the EXT family required for biosynthesis of HS chains, have decreased Wg, Hh, and BMP homologue Decapentaplegic (Dpp) protein levels and decreased signaling activity (Franch-Marro et al., 2005; Han et al., 2004a; Takei et al., 2004). Wing disc clones mutant for *sulfateless (sfl)*, which encodes the heparan sulfate *N*-acetylase/*N*-sulfotransferase, exhibit a similar reduction of protein level and signal activity of these three signaling ligands (Belenkaya et al., 2004a; Bornemann et al., 2004; Takei et al., 2004).

Classical studies of the effects of HS in development have focused on glypicans, heparan sulfate proteglycans (HSPG) anchored to the exterior cell membrane by glycosylphosphatidylinositol (GPI) (Lin, 2004). Glypicans are of particular interest for two reasons. First, glypicans play a role as signal cofactors and are involved in many organisms in the spreading and activity of Hh, Wnt, and BMP signals (Lin, 2004). Second, two of the essential structural components of glypican, heparan sulfate chains and GPI anchors, require UDP-GlcNAc for their synthesis. Two glypicans, Division-abnormally delayed (Dally) and Dally-like (Dlp), have been identified in Drosophila.

Among studies of GAGs, those of HS have been focused on its interactions with extracellular signaling and its roles in early development (Baeg et al., 2001, 2004; Desbordes and Sanson, 2003; Fujise et al., 2003; Han et al., 2004b; Rapraeger et al., 1985; Saunders et al., 1997). However, studies of CS have been mostly linked to roles in cartilage and bone development and health (Gould et al., 1992; Gualeni et al., 2010; Schwartz and Domowicz, 2002). Only more recently has focus on CS shifted towards its roles in early development.

The function of CS in developmental signaling

Many early studies of the role of GAGs in developmental processes focused primarily on the role of HS. Only in about the past 10 years has CS been the focus of developmental signaling research. A landmark study characterized the *sqv-5* (*squashed vulva-5*) gene in *C. elegans* (Mizuguchi et al., 2003). *sqv-5* encodes the *C. elegans* homologue of human Chondroitin synthase 1, which has alternating activity in adding GlcA and GalNAc onto the

growing CS polymer. Loss of function of *sqv-5* results in abnormalities in vulval development, possibly due to a requirement for CS in order to create a fluid-filled extracellular space.

C. elegans have large amounts of chondroitin deposited in oocytes, gonads, uterus, spermatheca, and fertilized eggs (Mizuguchi et al., 2003). (Note that this is unsulfated chondroitin, as C. elegans lack sulfotransferases necessary for chondroitin sulfation.) Additionally, chondroitin accumulates on the cell surface of cleavage-stage embyos (Mizuguchi et al., 2003). When a Chondroitin Synthase homologue is depleted by RNAi soaking or feeding, most oocytes and fertilized eggs die in utero, with escapers having low fertility and malformed gonads (Mizuguchi et al., 2003). At the six cell stage, embryos have a reversal of cell division following pseudocleavage, which leads to multinucleated cells and, ultimately, embryonic death. These data indicate that CS is required for proper cell division even as early the 4–6 stage of embryonic development. Some single-celled embryos with very low chondroitin sulfate fail to initiate cytokinesis, though nuclear division continues; the result is a single-celled embryo with an excess of 10 nuclei (Mizuguchi et al., 2003). Similar results were obtained when cultured embryonic cells were treated with chondroitinase ABC; cytokinesis was abnormal, and the cells with the greatest reduction in CS were likely to be polyploid (Mizuguchi et al., 2003). The *C. elegans squashed vulva* (*sqv*) genes *sqv-1* through *sqv-8* also play a role in HS and CS synthesis in *C. elegans*. Progeny of strong loss-of-function *sqv* homozygous mutants fail to initiate cytokinesis at the single celled embryonic stage (Hwang et al., 2003). CS loss cannot be solely responsible for these cytokinesis and polarity problems, and there is still much that must be learned about effects of CS on polarity in specific contexts. When Chinese hamster ovary cells were mutagenized to elimate HS and CS by targeting xylosyltransferase and galactosyltrasferase I, which are required to form the linker tetrasaccharide for CS and HS attachment, these cells did not have any defects in cytokinesis (Bai et al., 1999). Regardless, the work of Mizuguchi et al. set the stage for investigating the role of CS in early embryonic development and signaling.

CSPGs are highly expressed in the mammalian neural stem cell niche and play a key role in regulating cell fate and self-renewal (Sirko et al., 2007). When mouse embryonic neural stem cells are cultured to form neurospheres, their proliferation can be halted by addition of

Chondroitinase-ABC, which leads to an increase in astrocytes. In vivo, Chondroitinase-ABC injection into the telencephalic ventricle in intrauterine E13 embryos results in a decrease in self-renewing radial glia and a general reduction in neurogenesis, with accompanying increase in astrocytes. These results might indicate that CSPG plays a role in regulating neural stem cell growth and differentiation factors (Sirko et al., 2007).

HS-interacting factors may also interact with CS

There is considerable overlap between molecules that interact with heparin/HS and those that interact with CS; many molecules that are classically considered heparin/HS-binding have recently been shown to interact with CS and DS (Mizumoto et al., 2013). Indeed, hepatocyte growth factor is one HS-binding signaling factor shown to bind – and be activated by – DS and CS (Lyon et al., 2002). One study by Deepa, et al. (2002) examined the affinity to CS-E (purified from squid cartilage) of several growth factors known to bind to heparin (Deepa et al., 2002). Of all the growth factors tested (which included MK, PTN, FGF-1, FGF-2, HB-EGF, FGF-10, FGF-16, and FGF-18), only FGF-1 showed no affinity for CS-E. The affinity of MK, PTN, FGF-16, FGF-18, and HB-EGF for CS-E was equal to their affinity for heparin; this demonstrates that GAG chains can have both general affinity for growth factors, but in some cases the affinity is highly specific, as seen in the case of FGF-1.

It is, however, important to differentiate between signal binding and signal activation. A recent study investigated the abilities of lowly and highly sulfated chondroitin to interact with various growth factors and cytokines (Mizumoto et al., 2013). Though highly sulfated CS-E has a high affinity for VEGF and may play a role in vascularization of some cancers, exactly what that role is remains unclear (ten Dam et al., 2007). The coreceptor for VEGF, Neutrophilin-1, can have HS and/or CS attachment, dependent upon the context in which it is expressed. While both modifications result in Neutrophilin-1 having a high affinity for VEGF, only HS modification caused an increase in signaling. These data suggest that increasing CS may change Neutrophilin-1 from a coreceptor, which actively facilitates binding of VEGF to VEGFR2, to a pseudoreceptor or decoy, which binds and isolates VEGF from reception by VEGFR2 (Shintani et al., 2006). This

study highlights a unique method by which GAG balance can regulate VEGF signaling, as well as demonstrates that affinity for GAGs can have positive or negative effects on signaling.

CS interacts with molecules through many mechanisms

Popular models of CS-signaling protein interaction involve CS acting as a coreceptor, assisting in stabilizing molecules on the cell surface so that they may be accessed by their receptors (Figure 1.2A) or serving to titrate signaling molecules away from their receptors (Figure 1.2B). However, these are not the sole mechanisms whereby CS might affect extracellular signaling; the mechanisms by which CS could affect extracellular signaling are diverse. CS and other GAGs might stabilize signaling ligands and protect them from degradation (Figure 1.2C) (Akiyama et al., 2008). To enable FGF signaling, evidence suggests that GAGs assist in FGF dimerization (Figure 1.2D) (Ishihara et al., 1993; Walker et al., 1994), whereas for hepatocyte growth factor GAG binding induces a conformational change, which allows formation of a ternary signaling complex (Lyon et al., 2002). CS can influence signaling without interacting with a single signaling molecule. CS may function in order to alter the osmotic pressure in the extracellular matrix, causing expansion of the area there and altering diffusion of extracellular ligands (Figure 1.2E) (Comper and Laurent, 1978). There is also evidence that CS may drive morphogenesis embryonic sea urchin development independent of any signaling role, as the osmotic pressure to drive epithelial invagination during is driven by secreting CS into the extracellular matrix (Lane et al., 1993).

HS and CS synthesis are metabolically linked

In addition to overlap between binding targets, CS and HS themselves may be able to regulate the expression of the other. There is some evidence that syntheses of CS and HS are linked; i.e., inhibition of CS synthesis can result in upregulation of HS synthesis. Mice mutant for N-acetylgalactosaminyltransferase I, which catalyzes the transfer of the first GalNAc onto the tetrasaccharide linker, have an upregulation of Ext1 and Ext2, which transfer GlcNAc onto growing HS polymers. There was an accompanying increase in HS synthesis, but it is not clear if

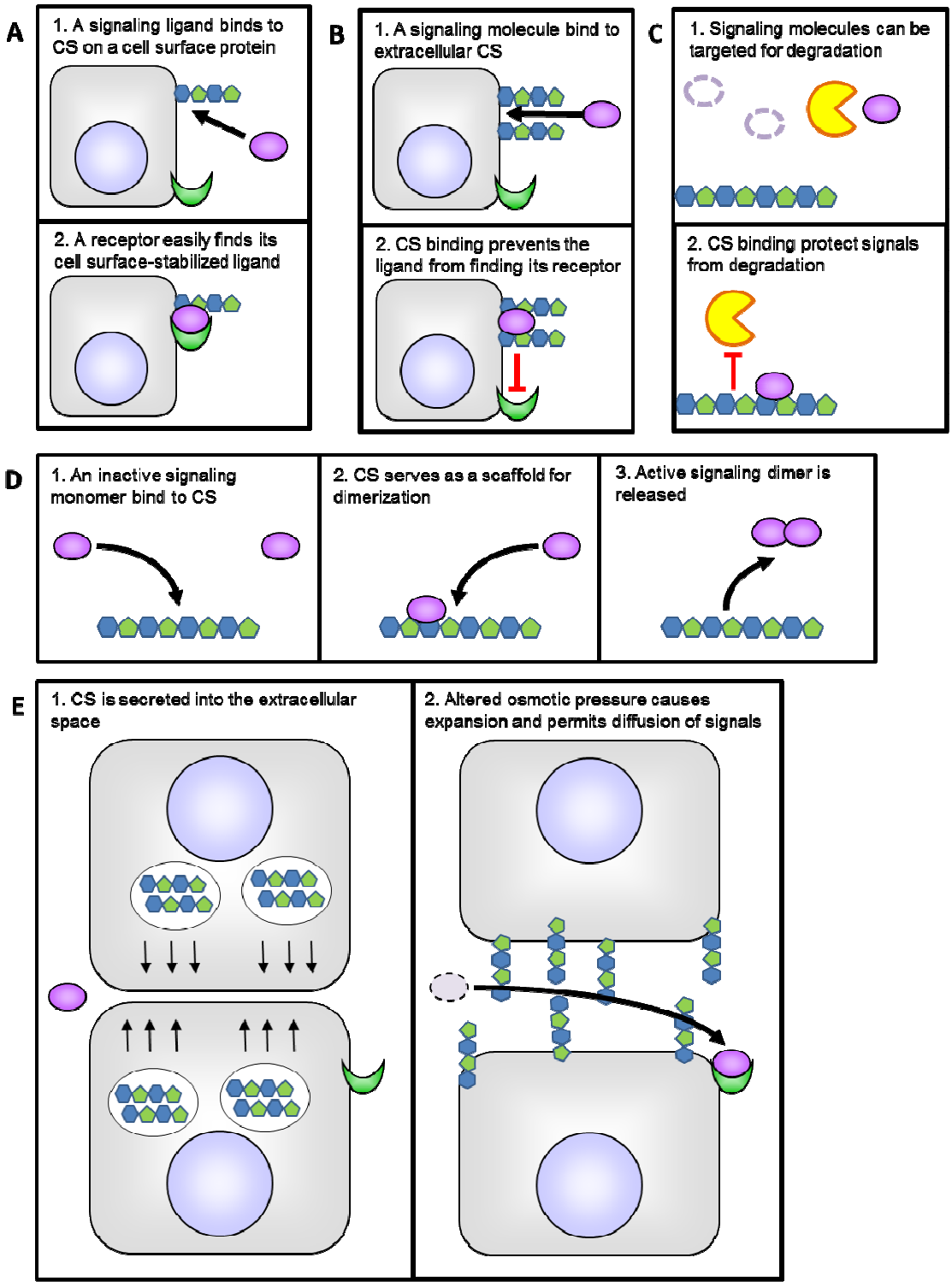

Figure 1.2. Models of signal regulation by CS. A) CS acts as a coreceptor to assist ligand-receptor interaction. B) CS binds and titrates the ligand away from its receptors. C) CS binding stabilizes the ligand and protects it from degradation. D) CS serves as a scaffold for assembly of ligand dimers or other signaling complexes. E. CS expands the space between cells, allowing passage of signaling ligands.

this increase was a result of the metabolic block of CS synthesis or if it was due to a separate effect of N-acetylgalactosaminyltransferase I in regulating Ext1 and Ext2 activity (Takeuchi et al., 2013). One possibility includes excess UDP-GalNAc resulting in activation of nutrient sensors that led to upregulation of Ext1 and Ext2 transcription or translation. It is notable that this increase in HS synthesis only occurred when CS synthesis was blocked, but not when CS was destroyed via chondroitinase ABC treatment, indicating that the shift is not due to a sensor of CS.

CS and HS have context-dependent biphasic activities

The effect of CS or HS on signaling in a particular tissue or organism may be the opposite in a different tissue or organism. However, the nature of signal regulation is organism- and tissue-specific; the glypican Dlp is positive for Hh signaling in *Drosophila* (Gallet et al., 2008), yet homologue Glypican-3 has been demonstrated to be a negative regulator of Hh signaling in mouse (Capurro et al., 2008). In Drosophila wing discs, glypicans are required for Dpp diffusion, and defects in HS synthesis lead to restriction of Dpp signaling (Fujise et al., 2003). In contrast, the *C. elegans* glypican Lon-2 has been identified as a negative regulator of BMP signaling (Gumienny et al., 2007).

The exact nature of these context-dependent effects of GAGs on signaling is unknown, but as previously noted it could be related to sulfation levels (Mizumoto et al., 2013) or to proteoglycan dosage. Dosage is very important in order for GAGs to have the proper effect on extracellular signals, and in many cases loss- and gain-of-function of a particular GAG can have a similar influence on signaling. In zebrafish embryos, *chsy1* loss of function (by morpholino injection) or gain of function (by injection of human CHSY1 mRNA) both lead to a similar defect in semicircular canal development (Li et al., 2010).

A study on the Xenopus *syndecan 1* (*xSdc1*) further supported this dosage model (Olivares et al., 2009). In particular, it was noted that injection of *xSyn1* mRNA into Xenopus embryos results in induction of a secondary axis; this phenotype is also observed when BMP signaling is reduced. To test if xSyn1 is acting as a BMP antagonist, *xSyn1* mRNA was injected into four-cell stage embryos, and P-Smad1 levels in the gastrula stage were evaluated. There

was a decrease in P-Smad1 in *xSyn1* mRNA injected embryos and also decreased levels of a luciferase reporter of BMP-induced transcription. Similarly, loss-of-function studies of *xSyn1* by morpholino injections resulted in a loss of luciferase reporter expression, suggesting deviation from endogenous xSyn1 levels results in BMP signal antagonism.

Epistasis experiments indicate that xSyn1 functions upstream of Smad1 and other intracellular components of BMP signaling, but downstream of BMP itself (Olivares et al., 2009). Interestingly, xSyn1 affects BMP signaling in a bell-shaped curve; when *xSyn1* morphant embryos are injected with *xSyn1* mRNA, low and high concentrations of mRNA have an inhibitory effect on BMP signaling, while moderate concentrations of mRNA restore BMP signaling. *xSyn1* mRNA is able to rescue embryonic dorsoventral patterning and BMP signaling even when its GAG attachment sites are mutated, indicating that this activity is carried out by the core protein itself and is independent of GAG (Olivares et al., 2009).

CS may play an unappreciated role in BMP signaling regulation

The two studies just described suggest that HSPG and CSPG can have biphasic effects on BMP signaling. In the *xSyn1* study, BMP signaling was antagonized when xSyn1 was either depleted or overexpressed, indicating a role for this HSPG in BMP signal regulation (Olivares et al., 2009). The inner ear phenotypes observed in zebrafish upon altering CHSY1 levels are also observed in response to Bmp2b gain of function and loss of function in the developing zebrafish ear (Li et al., 2010), suggesting a requirement for chondroitin sulfate to regulate Bmp signaling. In Drosophila, HSPGs have been well characterized in their ability to regulate the BMP homologue Dpp (Akiyama et al., 2008; Belenkaya et al., 2004a; Fujise et al., 2003; Jackson et al., 1997; Kirkpatrick et al., 2006a; Makhijani et al., 2007; Nakato et al., 1995; Navas et al., 2006). However, far less is known about the role of Drosophila CSPGs in Dpp regulation. In the following chapters I will present evidence indicating a role for CSPG in antagonizing Dpp signaling during Drosophila embryonic development.

CHAPTER 2

MUMMY, A UDP-N-ACETYLGLUCOSAMINE PYROPHOSPHORYLASE, MODULATES DPP SIGNALING IN THE EMBRYONIC EPIDERMIS OF DROSOPHILA

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Mummy, A UDP-N-acetylglucosamine pyrophosphorylase, modulates DPP signaling in the embryonic epidermis of Drosophila

Gregory B. Humphreys [1], Molly C. Jud [1], Kathryn M. Monroe [1], Suzanne S. Kimball, Matthew Higley, Danielle Shipley, Marie Clougherty Vrablik, Katherine L. Bates, Anthea Letsou *

Department of Human Genetics, Eccles Institute of Human Genetics, University of Utah, Salt Lake City, UT 84112, USA

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ABSTRACT

The evolutionarily conserved JNK/AP-1 (Jun N-terminal kinase/activator protein 1) and BMP (Bone Morphogenetic Protein) signaling cascades are deployed hierarchically to regulate dorsal closure in the fruit fly *Drosophila melanogaster*. In this developmental context, the JNK/AP-1 signaling cascade transcriptionally activates BMP signaling in leading edge epidermal cells. Here we show that the *mummy* (*mmy*) gene product, which is required for dorsal closure, functions as a BMP signaling antagonist. Genetic and biochemical tests of Mmy's role as a BMP-antagonist indicate that its function is independent of AP-1, the transcriptional trigger of BMP signal transduction in leading edge cells. pMAD (phosphorylated Mothers Against Dpp) activity data show the *mmy* gene product to be a new type of epidermal BMP regulator – one which transforms a BMP ligand from a long- to a short-range signal. *mmy* codes for the single UDP-N-acetylglucosamine pyrophosphorylase in *Drosophila*, and its requirement for attenuating epidermal BMP signaling during dorsal closure points to a new role for glycosylation in defining a highly restricted BMP activity field in the fly. These findings add a new dimension to our understanding of mechanisms modulating the BMP signaling gradient.

Introduction

The various forms of BMP (Bone Morphogenetic Protein) signaling are conserved throughout evolution. In organisms from flies to mammals, BMPs (which belong to the TGF-β [Transforming Growth Factor-β] superfamily of cytokines) function as essential patterning effectors: most notably specifying dorsoventral axis formation, maintaining stem cell niches in virtually all organisms, and directing cartilage and bone formation, fracture repair, joint maintenance and arthritic remodeling in vertebrates. The BMPs have also been implicated in pathologies ranging from neurodegeneration to fertility defects (Affolter and Basler, 2007; Dansereau and Lasko, 2008; Katsuno et al., 2011; Pogue and Lyons, 2006; Shimasaki et al., 2004). Despite these wide-ranging and essential BMP functions, many critical regulators of the pathway have yet to be elucidated. In particular, relatively little is known of BMP pathway modulation extracellularly, where the roles of proteins affecting receptor stability, ligand function, and ligand availability (such as proteoglycans, heparan and chondroitin sulfate modifying enzymes, and proteases) are only beginning to be identified and understood, likely due to their shared participation in signaling by multiple different ligands (Nishihara, 2010). The genetic and molecular studies of *mummy* (*mmy*), which we describe here, point to a role for the *mmy*-encoded UDP-N-acetylglucosoamine pyrophosphorylase as a BMP antagonist that acts directly in defining both the amplitude and range of the BMP signaling gradient. Within the context of dorsal closure in the fruit fly *Drosophila melanogaster*, the *mmy* gene product plays a central role in limiting embryonic epidermal BMP signaling. Moreover, Mmy function as an enzyme affecting protein modification by sugar attachment points to potential new targets for the treatment of BMP-associated developmental abnormalities and human disease pathologies.

Sequential JNK/AP-1 and BMP signaling activities direct dorsal closure

In Drosophila, dorsal closure occurs midway through embryogenesis when epidermal sheets, originally positioned ventrally and laterally, extend to the dorsal midline where they meet and fuse (reviewed in VanHook and Letsou, 2008). As the epidermis secretes the larval cuticle, dorsal-open group mutants remain uncovered by epidermis dorsally and accordingly secrete an incomplete cuticle that is distinguished by a large dorsal hole.

* Correspondence to: Eccles Institute of Human Genetics, 15 N. 2030 E., Rm. 2100, University of Utah, Salt Lake City, UT 84112. Fax: +801 581 7796.
E-mail address: aletsou@genetics.utah.edu (A. Letsou).
[1] These authors contributed equally to this manuscript.

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Table 1
Dorsal-open group loci encoding signaling molecules and their hierarchical relationships.

A. JNK signaling molecules	
slipper/slpr	JNKKK
hemipterous/hep	JNKK
basket/bsk	JNK
Jun related antigen/Jra	Jun transcription factor
kayak/kay	Fos transcription factor
slpr → *hep* → *bsk* → *Jra/kay* → gene expression (*dpp* and *puc*)	
B. Dpp signaling molecules	
decapentaplegic/dpp	TGF-β cytokine
thickveins/tkv	TGF-β type I receptor
punt/put	TGF-β type II Receptor
mothers against dpp/mad	Smad transcription factor
schnurri/shn	Zinc finger transcription factor
dpp→*tkv/punt*→*mad* and *shn*→gene expression	
C. *raw*-group signaling antagonists	
raw	novel
puckered/puc	MKP
ribbon/rib	BTB/POZ-type transcription factor
mummy/mmy	UDP-N-acetylglucosamine pyrophosphorylase

The process of dorsal closure is dependent upon changes in cell shape but not cell number; hence not unexpectedly, mutations in several cytoarchitectural molecules give rise to defects in dorsal closure. Many other dorsal-open group loci, however, code for components of the JNK/AP-1 (Jun N-terminal kinase/activator protein 1) or BMP signaling pathways, revealing these signaling cascades as integral to and essential for this fundamental morphogenetic event.

In dorsal closure, the JNK/AP-1 and BMP pathways act sequentially. First, AP-1 functions as the transcriptional activator of *dpp* (*decapentaplegic*; the Drosophila BMP homolog) in the dorsal-most row of epidermal cells: the leading edge (LE). Later, Dpp is thought to function in an autoregulatory fashion to maintain its own expression in the LE (Johnson et al., 2003). Consistent with these molecularly defined roles, loss-of-function mutations in activating components of the JNK/AP-1 and Dpp signaling cascades disrupt signaling and consequently dorsal closure. In JNK/AP-1 and Dpp signaling mutants (Table 1A,B), epidermal sheets fail to extend to and fuse at the dorsal midline (reviewed in Xia and Karin, 2004). At the opposite end of the spectrum is a small subset of dorsal-open group loci, termed the *raw*-group, that lead not to the absence of *dpp* in LE cells but rather to ectopic *dpp* in epidermal cells beyond the LE (Table 1C). Whereas loss of Dpp signaling leads only to dorsal cuticle holes, ectopic signaling leads to gross defects in ventral cuticle differentiation in addition to dorsal cuticle defects (Bates et al., 2008; Byars et al., 1999). Albeit clearly present, the ventral cuticle that is secreted from *raw*-group mutants is hypotrophic but neither mispatterned nor transformed, and conceptually at least, *raw*-group genes can function as regulators of either the JNK/AP-1 or Dpp signaling pathways.

Antagonizing signaling in embryonic dorsal closure

While our understanding of the JNK/AP-1 and Dpp signaling activators in dorsal closure is bolstered by molecular and biochemical studies in several systems, our understanding of the *raw*-group signaling antagonists is not as extensive. *puckered* (*puc*) is the best characterized of the three. *puc* codes for a VH1-like dual specificity protein tyrosine phosphatase belonging to the mitogen-activated protein kinase (MAPK) subfamily of MAP Kinase Phosphatases (MKPs) (Martin-Blanco et al., 1998). *puc* is required throughout the Drosophila life-cycle; one of its earliest functions is in LE cells during dorsal closure where it is transcriptionally activated by AP-1. It is thought that the Puckered MKP functions as a negative feedback regulator, dephosphorylating and inactivating Basket (Bsk), the JNK responsible for activating AP-1 in LE cells (Martin-Blanco et al., 1998).

The action mechanisms of the two additional dorsal closure signaling antagonists – *raw* (encoding a novel gene product; Byars et al., 1999) and *ribbon* (*rib*; encoding a BTB/POZ-type transcription factor; Bradley and Andrew, 2001; Byars et al., 1999; Shim et al., 2001) – have yet to be defined; although as is true for *puc*, *raw*-mediated effects on *dpp* are secondary to its effects on JNK/AP-1 signaling (Bates et al., 2008; Bauer Huang et al., 2007). Our previously published data (Bates et al., 2008; Byars et al., 1999) indicate that the *raw* gene product functions broadly in the epidermis to quench permissive AP-1 activity. Moreover, *raw*-dependent suppression of epidermal AP-1 sets the stage for LE-specific activation of AP-1 in LE cells of the epidermis.

GlcNAc regulation of Dpp activity

In the current report, we show that *mmy*, originally isolated in the Heidelberg screen for embryonic lethals affecting cuticle pattern (Nusslein-Volhard et al., 1984), represents the newest member of the *raw*-group of signaling antagonists. Within this group, however, *mmy* function is unique. In contrast to the *raw* and *puc* gene products, which restrict the signaling domain of Dpp secondarily through their modulation of JNK/AP-1, *mmy's* effects upon Dpp signal transduction are direct.

mmy codes for the single Drosophila UDP-N-acetylglucosamine pyrophosphorylase, a key enzyme in UDP-N-acetylglucosamine (UDP-GlcNAc) biosynthesis (Araujo et al., 2005; Schimmelpfeng et al., 2006; Tonning et al., 2006). It is clear from previous reports that *mmy* is required in Drosophila for the synthesis of extracellular chitin (an insoluble polymer of GlcNAc), and that this requirement manifests itself as cuticular and tracheal defects in strong loss-of-function *mmy* mutants (Araujo et al., 2005; Devine et al., 2005; Tonning et al., 2006). Chitin synthesis is, however, unaffected in certain *mmy* hypomorphs including those exhibiting defects in dorsal closure, and thus the dorsal-closure defects observed in animals homozygous for these *mmy* hypomorphs are thought to result from another requirement for UDP-GlcNAc (Araujo et al., 2005; Schimmelpfeng et al., 2006; Tonning et al., 2006).

In addition to being the building block of chitin, UDP-GlcNAc is an essential precursor for the synthesis of heparin and chondroitin sulfate proteoglycans, the former having been shown to play an essential role in modulating the effects of Dpp/BMP, Wingless (Wg)/WNT, and Hedgehog (Hh) morphogen signaling in Drosophila and other eukaryotes, usually as a facilitator of long-range signaling (Akiyama et al., 2008; Beckett et al., 2008; Belenkaya et al., 2004; Capurro et al., 2008; Gallet et al., 2008; Gumienny et al., 2007). Evidence presented in the current report points to a new role for GlcNAcylation in modulating Dpp activity. Our results demonstrate that Mmy/UDP-GlcNAc constrains rather than facilitates epidermal Dpp signaling during closure, presumably by limiting the signaling capacity of the Dpp cytokine that is produced in LE epidermal cells.

Materials and methods

Drosophila strains

Fly lines for this study include *mmy*^{1}, *mmy*^{P15133}, *raw*^{1}, *aop*^{1}, *bsk*^{2}, *Jra*^{IA109}, *puc*^{H246}, *rib*^{1}, *Df(2L)BSC6*, *UAS-brk* and 69*B-gal*4 (Marygold et al., 2013), as well as *mmy*^{LM1}, *mmy* ^{LM16}, *mmy*^{LM24}, *mmy*^{LM45}, *mmy*^{LM47} and *mmy*^{LM51} (M. Krasnow), and *puc*^{E69} (A. Martinez-Arias).

Phenotypic analyses

Embryonic lethal cuticle phenotypes were viewed after mounting samples in one-step mounting medium (30% CMCP-10, 13% lactic acid, 57% glacial acetic acid). For hybridizations in situ, we used digoxigenin-labeled RNA as described (Byars et al., 1999); with mouse anti-digoxigenin alkaline phosphatase or mouse anti-digoxigenin (Roche). For immunostains, we used rabbit anti-Jun (Santa Cruz Biotechnology), rabbit anti-Phospho-Smad1,5 Ser463/465 (Cell Signaling Technology), mouse anti-β-Gal (Promega), goat anti-mouse alkaline phosphatase (Promega), goat anti-rabbit alkaline phosphatase (Jackson ImmunoResearch), and goat anti-rabbit Alexa Fluor 488 antibodies (Invitrogen Molecular Probes).

RT-PCR

Mutant homozygotes were distinguished from wild-type siblings 4–8 and 8–12 h AEL (after egg lay) based on the absence of a GFP (Green Fluorescent Protein)-marked balancer chromosome. RNA was isolated from wild-type and mutant embryos, and reverse transcripts generated using a dT_{16} primer. PCR products were generated using *RA*- and *RB*- specific 5′primers in combination with an exon two 3′primer.

Protein studies

For immunoblotting studies, proteins were prepared from experimental and control lysates 8–12 h AEL. Mutant homozygotes were distinguished from wild-type siblings based on the absence of a GFP-marked balancer chromosome. CIP (Calf Intestine Alkaline Phosphatase) was added to half-portions of each lysate. Treated and untreated lysates were separated on SDS-acrylamide gels and analyzed by western blotting using anti-Jun antibody. The secondary antibody was HRP-conjugated goat anti-rabbit antibody (Chemicon). Jun densitometry values were calculated as ratios of experimental to control band integrated intensities (the product of mean band intensity and pixel number in an inverted image) and normalized to wild-type CIP-treated and untreated ratios. Similar methods were used to quantify pMAD in the dorsal lateral epidermis of wt and *mmy*^{1} embryos. The number of pMAD-positive nuclei in the dorsolateral epidermis were counted in columns of cells in T1, T3, A4 and A6 in wt and *mmy*^{1} embryos (n=14 and 11 embryos, respectively). pMAD-positive nuclei were counted in the middle and posterior of each segment in all embryos. Short (middle) and long (posterior) column depths were averaged for each embryo.

Yeast transformation and rescue

Rescue studies were performed in *qri*1 *Saccharomyces cerevisiae*. Diploid *ura*3 yeast strains harboring a heterozygous KanMX4 insertion in *QRI*1 were transformed with the *mmy-RA*$^{+}$ *URA*3 expression plasmid (constructed by insertion of the full-length *mmy RA* cDNA into p426-ADH1). After transformation, yeast cells prototrophic for uracil were induced to sporulate by standard methods and tetrads dissected by micromanipulation. Rescue of *qri*1 lethality was determined by assessing growth on CM-ura, YPD +G418, and YPD+5-FOA agar plates.

Results

Mummy functions as an N-acetylglucosamine pyrophosphorylase

The Drosophila genome encodes a single UDP-N-acetylglucosamine pyrophosphorylase (*mummy* [*mmy*]), which potentially functions in the synthesis of saccharides as well as the modification of multiple glycosylated protein and lipid products (Fig. 1A). Two transcript isoforms (*RA* and *RB*) are derived from the *mmy* locus. The *RB* isoform is homologous along its entire length to the eukaryotic family of UDP-N-acetylglucosamine pyrophosphorylases; the *RA* isoform, in contrast, encodes a distinctive amino terminus, the result of a splice form variant created by use of an alternative 5′ exon (Fig. 1B). The *RA* amino terminus comprises a 37 amino acid stretch that has yet to be identified in genome scans of any sequenced organism other than members of the genus *Drosophilidae*. Except for the species *willistoni*, the sequence is highly conserved in all members of the subgenus *Sophophora* (Fig. 1C).

While our current understanding of the genome does not allow us to predict how the unique 5′ end of the *mmy RA* isoform affects its enzymatic function, we show here that the *RA*-encoded protein product retains N-acetylglucosamine pyrophosphorylase activity in a heterologous rescue assay. A plasmid with the Drosophila *mmy*$^{RA+}$ gene fully restores viability to a *S. cerevisiae* strain with a disruption in the essential *QRI*1 gene (the yeast *mmy* homolog; Fig. 1D). A diploid *ura*3 yeast strain harboring a heterozygous KanMX4 insertion in *QRI*1 was transformed with the *mmy-RA*$^{+}$ *URA*3 expression plasmid. Transformation resulted in uracil prototrophy, as indicated by *qri*1^{+} and *qri*1^{-} spore survival on complete medium lacking uracil (CM-ura). Whereas transformed *qri*1^{+} spores cannot grow on medium supplemented with the drug G418 (YPD+G418), transformed *qri*1^{-} spores grow well in the presence of G418, indicating that the KanMX4 insertion remains in *qri*1^{-} spores after transformation. Finally, transformed *qri*1^{-}, but not *qri*1^{+}, spore viability is dependent upon the presence of *mmy-RA*$^{+}$ *URA*3 expression plasmid as only *qri*1^{+} spores survive exposure to 5-FOA (which elicits plasmid loss). Together, these data indicate that *qri*1^{-} viability is dependent upon presence of the *mmy-RA*$^{+}$ *URA*3 expression plasmid and show that the Drosophila *mmy* RA gene product is an orthologue of the yeast N-acetylglucosamine pyrophosphorylase QRI1. Thus, as is the case for the human and *Candida albicans QRI*1 loci (51% and 41% identical and 70% and 60% similar to Drosophila *mmy,* respectively), the novel Drosophila *mmy RA* isoform rescues the lethal phenotype associated with the *S. cerevisiae qri*1 null mutation

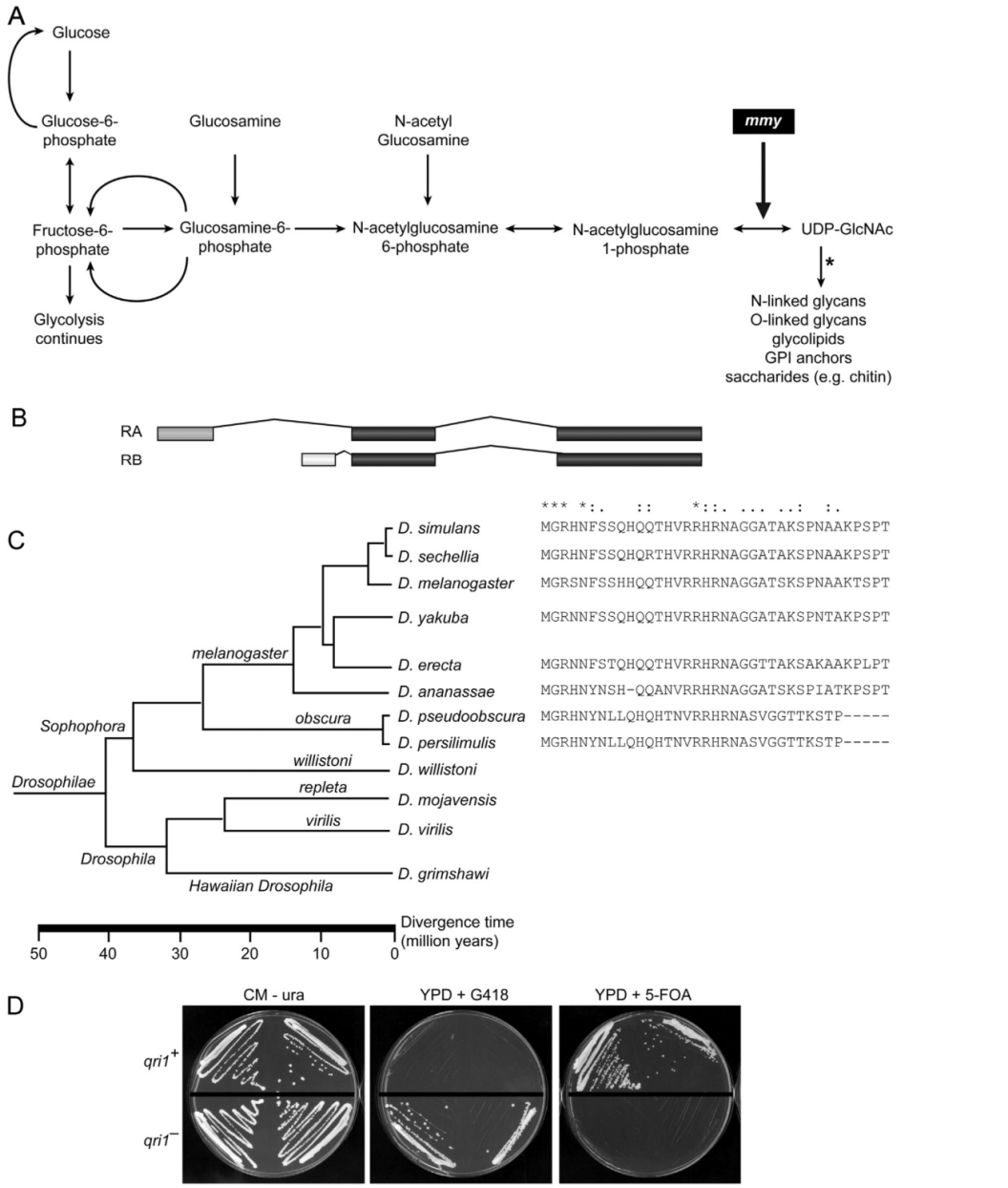

Fig. 1. Organization and expression of the *mmy* gene. (A) Schema for the generation of UDP-GlcNAc from glucose. In Drosophila there are at least 26 transferases (Correia et al., 2003), each of which can catalyze the transfer of GlcNAc from UDP-GlcNAc to a subset of acceptors. The transferase-dependent step is marked (*). (B) Intron-exon map of *RA* and *RB mmy* isoforms. (C) Cladistic analysis of the protein sequences encoded by *RA* exon 1. (D) A diploid *ura3* yeast strain harboring a heterozygous KanMX4 insertion in *QRI1* was transformed with the *mmy-RA*$^{+}$ *URA3* expression plasmid; the growth properties of four spores derived from a single tetrad are shown.

affecting N-acetylglucosamine pyrophosphorylase activity (Mio et al., 1998).

mmy expression is dynamic in Drosophila embryos

In addition to employing alternative first exons, *RA* and *RB mmy* isoforms also differ in their transcriptional regulation. Whereas *RA* transcript levels are invariant in wild-type embryos, *RB* transcript levels are low in wild-type embryos 4–8 h AEL but greatly elevated a short time later, 8–12 h AEL (Fig. 2A). During this period, the embryo develops trachea and undergoes dorsal closure. Despite conflicting descriptions of *mmy* expression in the literature (Araujo et al., 2005; Tonning et al., 2006), our strictly temporal analysis of *mmy* gene expression in RT-PCR studies provides clear

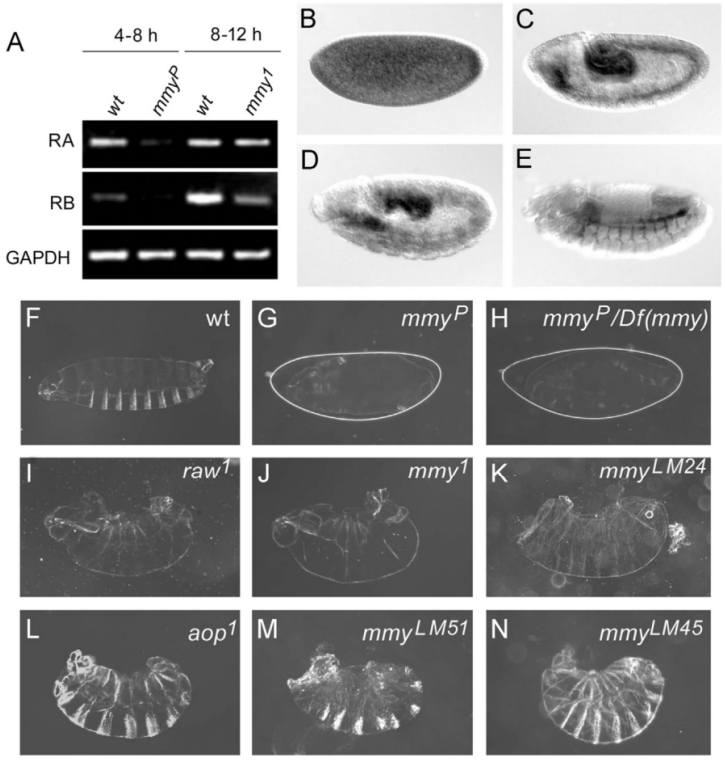

Fig. 2. Pleiotropic effects of *mmy*. (A) Temporal profile of *mmy* transcription in early embryogenesis. *RA* and *RB* transcripts were distinguished and quantified by RT-PCR using RNAs isolated from wild-type and mutant (*mmy*^{1}and *mmy* ^{P15133}, here abbreviated *mmy*^{P}) embryos 4–8 and 8–12 h AEL. GAPDH RNA levels are shown as a control for loading. (B–E). Spatial profile of *mmy* transcription in hybridizations to whole mount embryos in situ using a digoxigenin-labeled *mmy* RNA as probe: (B) syncytial blastoderm, (C) germ band extension, (D) germ band retraction, and (E) dorsal closure. In contrast to the asymmetrically patterned cuticle that is derived from (F) wild-type embryos, (G) *mmy*^{P15133} homozygotes and (H) *mmy*^{P15133}*/Df (3R)*345 transheterozygotes fail to secrete cuticle (and the preps have not been devitellinized). Hypomorphic *mmy* mutations lead to shared loss-of-function phenotypes with mutations in genes that modulate JNK and/or Dpp signaling pathways: (I) *raw*^{1}, (J) *mmy*^{1}, (K) *mmy*^{LM24}, (L) *aop*^{1}, (M) *mmy*^{LM51}, and (N) *mmy*^{LM45}.

evidence that *mmy* is expressed early in Drosophila embryogenesis. Complementing our temporal expression data are spatial expression data derived from in situ hybridization studies (Fig. 2B–E). These studies extend previously published reports (Araujo et al., 2005; Tonning et al., 2006) in revealing *mmy* expression to be dynamic spatially, as it is temporally, throughout embryogenesis. We found that *mmy* is expressed ubiquitously and uniformly in the cellular blastoderm. However, even though ubiquitous *mmy* expression persists throughout embryogenesis, transcript accrual at later developmental time points is spatially partitioned. We noted *mmy* accumulations in the developing mesoderm, gut primordia, and trachea. Overall, widespread *mmy* expression reveals its potential to function in multiple aspects of Drosophila embryonic development, consistent with its role as the single UDP-N-acetylglucosamine pyrophosphorylase in Drosophila.

mmy is essential for multiple developmental events in Drosophila

The sequence and regulatory differences that characterize the two *mmy* transcripts, as well as the spatially broad and dynamic *mmy* expression profile, suggest that *mmy* function is pleiotropic. Although only the *mmy*-dependent chitin defects have been characterized in detail (Araujo et al., 2005; Tonning et al., 2006), results from our genetic studies are consistent with the idea that the *mmy*-encoded N-acetylglucosamine pyrophosphorylase impacts multiple Drosophila developmental events via the action of several different downstream transferases, some of which modify proteins and lipids with GlcNAc (see Fig. 1A). Independently-derived *mmy* mutants exhibit a variety of highly penetrant phenotypes, ranging from cuticle defects associated with a failure to synthesize chitin (Fig. 2F–H) to cuticle defects associated with well-characterized Dpp-dependent closure abnormalities (dorsal closure and head involution; Fig. 2I–N). In particular, these *mmy*-associated cuticle defects are identical to those resulting from loss-of-function mutations in *raw* and *anterior-open* (*aop*), and point to a crucial role for Mmy in regulating embryonic Dpp signaling.

Absence of cuticle is thus far the best characterized of the *mmy* phenotypes and clearly results from deficiencies in chitin synthesis (Araujo et al., 2005; Schimmelpfeng et al., 2006; Tonning et al., 2006). We have used two independent strategies to show that the cuticleless phenotype defines the strongest loss of zygotic function condition. First, we confirmed that the cuticleless *mmy*^{P15133} allele harbors a transposon insertion within *mmy*'s second intron and demonstrated that the insertion affects transcription of the *RA* and *RB* transcripts, both being markedly reduced in *mmy*^{P15133} homozygotes in comparison to wild-type controls (Fig. 2A). More notably, we demonstrated that the *mmy*^{P15133}*/mmy*^{P15133} embryonic lethal cuticular phenotype is

indistinguishable from that of $mmy^{P15133}/Df(2L)BSC6$ transheterozygotes and thus genetically defined the mmy^{P15133} allele as null (Fig. 2G,H).

While there is no redundancy in the biosynthetic pathway leading to the generation of UDP-GlcNAc, GlcNAc itself is distributed to distinct protein targets via the action of several different downstream UDP-GlcNAc transferases. Hence, even as the *mmy* null condition reveals much about the central role for GlcNAc in chitin synthesis, it likely masks other equally important, albeit independent, *mmy*-dependent GlcNAcylation processes. To help us understand one of these additional roles for *mmy* and GlcNAc more fully, we turned our attention to the Class III *mmy* mutants that as a group were characterized previously as strong loss-of-function mutants sometimes associated with dorsal closure defects (Devine et al., 2005). mmy^1, a focus of the studies described here, is predicted from sequencing studies to be a regulatory mutant; indeed, the *RB* transcript is specifically affected, showing a 3-fold reduction 8–12 h AEL, corresponding to the time when mmy^1 mutants abort development due to defects in dorsal closure (Fig. 2A). Our sequencing studies revealed that the mmy^1 coding region harbors neither missense nor nonsense mutations (data not shown). Other *mmy* alleles showing a high penetrance of dorsal closure and ventral cuticle defects are mmy^{LM1}, mmy^{LM16}, and mmy^{LM24}. Respectively, these alleles harbor missense mutations in the *mmy*-encoded N-acetylglucosamine pyrophosphorylase substrate-binding site (S204L) and the diphosphorylase consensus motif (G150S; G148R) (Devine et al., 2005).

Mmy antagonizes Dpp signaling

The *mmy* dorsal-open, ventral-hypotrophic cuticle phenotypes (see Fig. 2J,K) led to our speculation that Mmy-dependent glycosylation might be integral to restriction of Dpp signaling during closure. In particular, we noted that in all closure-defective *mmy* mutants, dorsal closure defects do not appear in isolation but rather are associated with ventral cuticular defects that we and others have shown previously to be associated with ectopic Dpp (Bates et al., 2008; Byars et al., 1999; Riesgo-Escovar and Hafen, 1997). We employed molecular and functional experimental strategies to test the hypothesis that Mmy is required to limit Dpp signaling in the embryonic epidermis of Drosophila.

First, we directly visualized embryonited [activated] form of the Dpp signal transc epidermal Dpp activity. To do this, we used an antibody directed against pMAD (the phosphoryladucer Mothers against dpp) in conjunction with both DIC (differential interference contrast) and confocal imaging methods. Using DIC, we detected pMAD very broadly in the epidermis of wild-type embryos undergoing germ band extension (Fig. 3A); later in development (in germ band retracting stages of embryogenesis), we observed diminution of the pMAD immunoreactive domain (Fig. 3C). Attenuation of the Dpp signaling amplitude is most evident at dorsal closure. We detected pMAD staining at levels only modestly above background in the dorsal epidermis of 48% of dorsal-closure stage embryos and no pMAD in the dorsal epidermis of 52% of dorsal-closure stage embryos ($n=219$; Fig. 3E,G). Our pMAD immunoreactivity profile data point to a previously unrecognized tissue-specific Dpp signaling transition in the epidermis of wild-type, dorsal-closure stage embryos – from widespread and robust in germ band extended stages to restricted and then undetectable in dorsal closure stages.

When we examined the epidermal Dpp signaling domain in *mmy* mutant embryos, we found that although germ band extended and retracted pMAD profiles are similar in wild-type and *mmy* mutant embryos (Fig. 3B,D), differences are evident later in development. Dpp signaling, which is attenuated in dorsal closure stages of wild-type embryogenesis, persists temporally

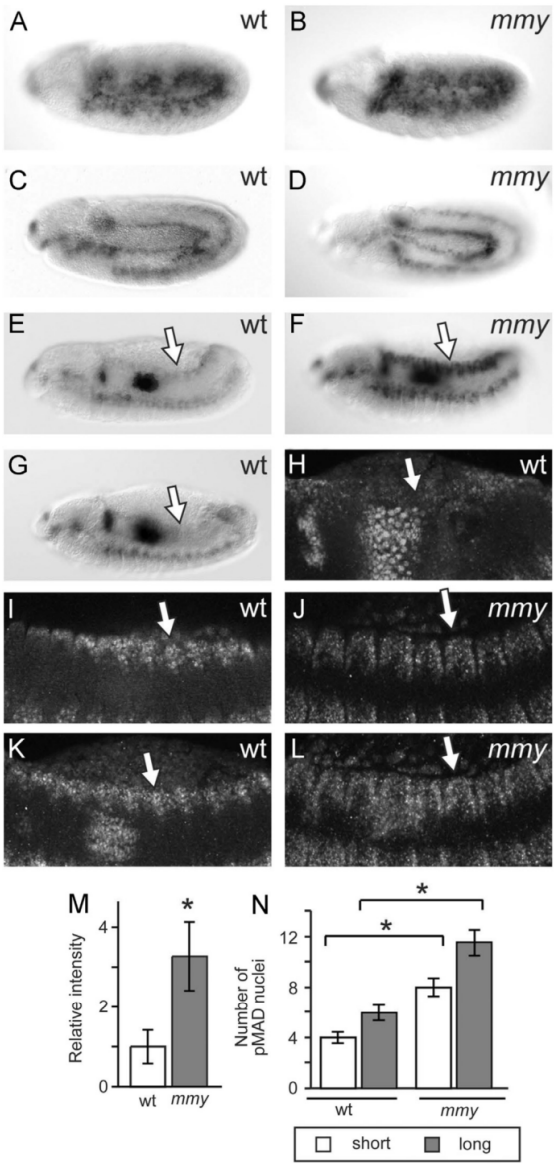

Fig. 3. *mmy*-mediated restriction of Dpp signaling during closure. pMAD immunolocalization in whole mount wild-type (wt) and mmy^1 (*mmy*) mutant embryos in (A,B) germ band extended, (C,D) germ band retracting, and (E–G) dorsal closure stages of embryogenesis. (H–L) Immunolocalization of pMAD in wild-type (wt), and mmy^1 (*mmy*) embryos imaged by laser scanning microscopy with (I,J) representing single focal planes and (K,L) corresponding to compilations of multiple Z-stacks. (M) Quantitation of epidermal pMAD immunostain intensities in wt and *mmy* embryos at dorsal closure. Values were calculated as experimental ($n=17$ for wt and $n=13$ for *mmy*) to wt ratios of background-corrected integrated intensities in inverted images of whole-mount embryos. * indicates that the calculated wt and *mmy* values are statistically different ($p=7\times10^{-6}$), while error bars correspond to the confidence interval of the mean ($p<0.05$). (N) Quantitation of spatial extent of pMAD immunostain in the epidermis of wt and *mmy* embryos at dorsal closure; measurements were obtained from the shortest (middle) and longest (posterior) pMAD-staining region of each segment. * indicates the calculated wt and *mmy* values are statistically different ($p<1.6\times10^{-10}$), while error bars correspond to the confidence interval of the mean ($p<0.05$).

and extends spatially in similarly staged *mmy* mutants (Fig. 3F). In 100% of dorsal-closure stage *mmy* embryos, pMAD remains robustly expressed in the dorsal epidermis at levels ~3-fold higher than that observed in the pMAD-positive fraction of wild-type embryos (Fig. 3M). Analysis of wild-type and mutant embryos by confocal microscopy not only validated the genotype-associated spatio-temporal differences in Dpp signaling that we documented previously by DIC microscopy, but also provided a platform for quantification of spatial differences (Fig. 3H–L,N). We observed that while pMAD immunoreactivity can extend to an average depth of five epidermal cells in some dorsal closure stage wild-type embryos, immunoreactivity extends to an average depth of ten epidermal cells in all similarly staged *mmy* mutants.

Having established that pMAD persists broadly in the epidermis of dorsal closure stage *mmy* embryos, we next applied functional tests to assess whether Dpp gain-of-function is causative of developmental abnormalities in *mmy* mutants. To this end, as epidermal Dpp can be autoregulatory (Arora et al., 1995; Johnson et al., 2003), we compared epidermal *dpp* expression profiles in wild-type and mutant (mmy^1 and mmy^{LM16}) whole mount embryos in situ. In wild-type embryos, from germ band extended to germ band retracted stages of development, we observed epidermal *dpp* only in LE cells (Fig. 4A–C). In contrast, in similarly staged dorsal-open *mmy* mutants we observed ectopic *dpp* transcription in the embryonic epidermis (Fig. 4D–F), analogous to that which we documented previously in *raw* and *raw*-group mutant embryos (Fig. 4L; see also Bates et al., 2008). Thus, as for other members of the *raw*-group, in *mmy* mutants a poorly differentiated cuticle is linked to expansion of the epidermal *dpp* expression domain.

Next, we employed the UAS-GAL4 system to express the well-characterized *dpp* antagonist *brk* in the ectopic epidermal Dpp signaling domain of *mmy* mutants (Brand and Perrimon, 1993; Scuderi and Letsou, 2005). Cuticles derived from mmy^1/mmy^1; *UAS-brk/69B-gal4* transgenics revealed rescue of mmy^1-dependent defects; particularly clear was the restoration of ventral denticles to the cuticle (Fig. 4O; see also Fig. 2J for mmy^1/mmy^1 mutant comparison). It is notable that the *brk* and *dpp* domains are neither overlapping nor abutting in wild-type embryos undergoing dorsal closure (Jazwinska et al., 1999). Moreover, the *brk* expression domain is not altered in *mmy* mutants (Fig. 4M,N). Together, our Dpp/*dpp* localization and *brk* suppression data show that Dpp activity expands in *mmy* mutants and is causative of developmental abnormalities.

Mmy modulation of Dpp signaling is Dpp-dependent and AP-1-independent

We next sought insight into the molecular basis of Mmy-mediated antagonism of Dpp signaling. Given our understanding of Dpp/*dpp* regulation during dorsal closure as well as in other developmental contexts, we speculated that *mmy* might restrict epidermal Dpp signaling: (1) in an established manner (e.g. like *raw* and *puc*), transcriptionally via JNK/AP-1-mediated restriction of *dpp* gene activation, or (2) in a novel manner (with respect to the LE and epidermis), post-transcriptionally via restriction of autoregulatory Dpp signaling activity.

We employed several strategies to discriminate between transcriptional and post-transcriptional models of Mmy function as a Dpp signaling antagonist. First we assayed whether ectopic

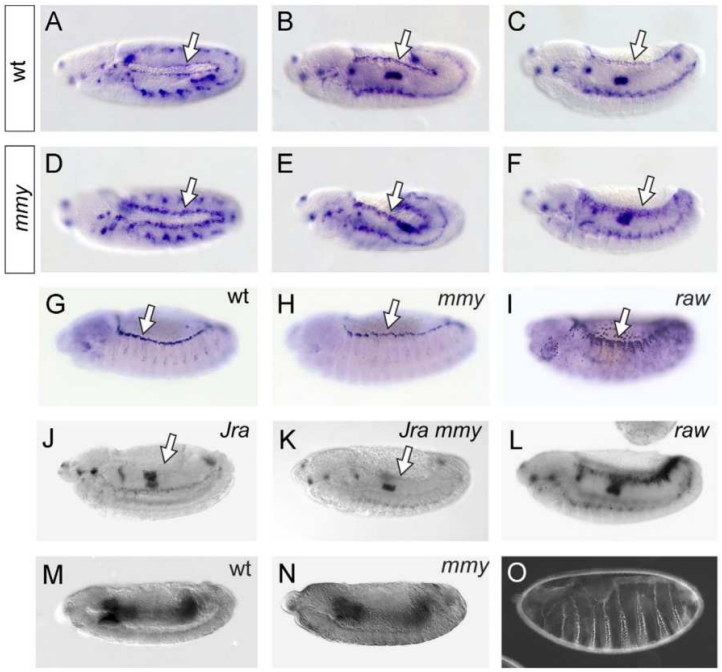

Fig. 4. Ectopic Dpp signaling in *mmy* mutants is causative of developmental abnormalities. *dpp* mRNA transcript expression in wild-type (wt) and mutant (mmy^1) whole mount embryos: (A,D) germ band extended, (B,E) germ band retracting, and (C,F) dorsal closure stages of embryogenesis. *dpp* expression is also shown in (J) Jra^{IA109}, (K) Jra^{IA109} mmy^1, and (L) raw^1, dorsal closure stage embryos. Expression of the puc^{E69} enhancer trap in (G) wild-type, (H) mmy^1, and (I) raw^1 mutant embryos during dorsal closure. (M,N) *brinker* expression in wild-type and mmy^1 homozygotes; (O) rescued ventral cuticle in mmy^1; *UAS-brk/69B-Gal4* transgenics. In all panels, the LE is indicated with an arrow.

epidermal gene expression in *mmy* mutants is limited to *dpp* or whether other transcriptionally regulated LE targets of JNK/AP-1 are ectopically expressed as well. In addition to *dpp*, there is another well-characterized transcriptionally-regulated target of JNK/AP-1 activation in the LE during closure – *puc*. As is true for *dpp*, *puc* transcription is abolished in LE epidermal cells in embryos harboring mutations in JNK/AP-1 signaling activators, including *hep* (JNKK), *bsk* (JNK), and *Jra* (Jun) (Glise and Noselli, 1997), and expanded in embryos mutant for JNK signaling antagonists *raw* and *puc* (MKP) (Byars et al., 1999; Ring and Martinez Arias, 1993).

We used the well-characterized JNK/AP-1 responsive *puc* enhancer trap (puc^{E69}-*lacZ*) to monitor LE *puc* expression. As we and others have reported previously, we observed β-Gal activity that temporally and spatially mirrors LE *dpp* expression in wild-type animals (Byars et al., 1999; Dobens et al., 2001; Stronach and Perrimon, 2001); in this regard, we detected β-Gal initially in the LE of germ band extended embryos and we observed its persistence in LE cells throughout dorsal closure stages of development (Fig. 4G). In *mmy* mutants, β-Gal never expanded beyond the LE epidermal domain as it does in *raw* and *puc* mutant embryos (Fig. 4H,I; see also Byars et al., 1999). Our observation that *mmy*-mediated expansion of *dpp* gene expression does not extend to a second transcriptionally-regulated target of JNK/AP-1 in LE cells (*puc*) suggests that ectopic *dpp* transcription in *mmy* mutants is not a consequence of ectopic AP-1 activity.

Next, we employed biochemical methods to examine Jun in wild-type and mutant embryos directly. For initial measures of Jun/AP-1 activity in wild-type and *raw*-group mutant embryos, we identified an anti-Jun antibody that recognizes endogenous Drosophila Jun protein in extracts isolated from wild-type embryos (Fig. 5A). In comparisons of phosphatase-treated and untreated lysates, we identified phosphorylated (activated) and unphosphorylated (inactivated) Jun isoforms (Peverali et al., 1996). In wild-type embryos, both the phosphorylated and unphosphorylated isoforms are present (Fig. 5B). Immunoblotting studies indicated that the wild-type balance between phosphorylated and unphosphorylated Jun isoforms is unchanged in *mmy* mutants (Fig. 5C), demonstrating that overall embryonic phosphorylated and unphosphorylated Jun levels are not detectably altered in this quantitative assay. In contrast though, levels of phosphorylated and unphosphorylated Jun are altered considerably in *raw* mutants (Fig. 5D). Not only is the unphosphorylated Jun isoform undetectable in non-CIP treated extracts from raw^1 (null) mutant embryos, but the more slowly migrating phosphorylated isoform accumulates to measurably higher levels in raw^1 and raw^1 bsk^2 double mutant embryos than it does in wild-type embryos (more than 2-fold for both). This observation, as well as a parallel immunoblotting analysis of extracts from bsk^2 nulls revealing a pattern of Jun phosphorylation that is indistinguishable from that of wild type, is consistent with our previous strictly genetic prediction that the majority of Jun phosphorylation in wild-type embryos is dependent upon a Jun kinase other than zygotic Basket, and that Raw antagonizes the function of this kinase (Bates et al., 2008).

Immunolocalization studies in *mmy* and *raw* mutant embryos complemented western studies and further bolstered our conclusion that Raw mediates *dpp* antagonism via its function upstream of Jun, while Mmy-mediated *dpp* antagonism likely occurs downstream. From immunolocalization studies, it is clear that: (1) Jun is expressed broadly in wild-type and mutant (mmy^1 and raw^1) embryos, and (2) Jun does not differentially accumulate in LE cells from *raw* embryos as it does in *mmy* and wild-type dorsal-closure stage embryos (Fig. 5E–H). Thus, biochemical markers of phenotype, in conjunction with molecular markers, indicate that *mmy* is

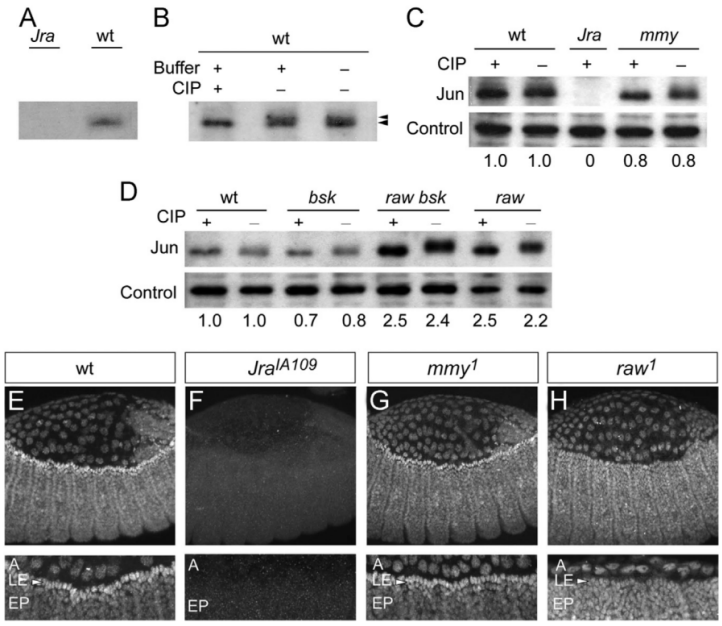

Fig. 5. JNK signaling is normal in *mmy* mutants, but not in *raw* mutants. (A) Anti-Jun antibody recognizes wild-type protein but not a truncated version lacking the epitope derived from Jra^{IA109} mutants. (B) Anti-Jun antibody recognizes a doublet in untreated extract but a singlet in extract treated with CIP. Jun modification and accumulation in (C) mmy^1, and (D) raw^1 and bsk^2 mutant embryos. Total Jun protein was quantified, and measurements relative to wild-type are reported at the bottoms of panels C,D. Controls for protein loading equivalency are shown for all gels where protein was quantified. Immunolocalization of Jun in (E) wild-type, (F) Jra^{IA109}, (G) mmy^1, and (H) raw^1 embryos imaged by laser scanning microscopy with no (top row) and 2.5x (bottom row) zoom. In 2.5x zoomed images, A denotes Amnioserosa, LE denotes leading edge epidermis, and EP denotes epidermis.

different from other members of the raw group. While *raw* and *puc* antagonize Dpp indirectly through their function as Jun pathway modulators, the effects of *mmy* are downstream of the Jun pathway and likely target the Dpp pathway directly.

Finally, having established that *mmy*-dependent defects in closure effects are Jun-independent, we used mutants in the JNK/AP-1 signaling pathway to specifically ablate LE *dpp* and to test whether *mmy*-dependent defects in dorsal closure are dependent upon LE *dpp*. While we detect *dpp* ectopically in the epidermis of *mmy* mutant embryos, its expression is absent from the epidermis of *mmy Jra* and *mmy bsk* double mutants as it is also in *Jra* and *bsk* single mutant backgrounds (Fig. 4J,K and data not shown). Our demonstration that null mutations in either *Jra* or *bsk* prevent manifestation of the ectopic *dpp* phenotype associated with mmy^{1} indicates that Jun is the lone transcriptional trigger of epidermal *dpp* in both wild-type and *mmy* mutant embryos, and even more notably that LE JNK/AP-1-dependent *dpp* expression is a prerequisite for the expansion of epidermal *dpp* that we observe in dorsal-open *mmy* mutants. Thus, *mmy*'s effects are Dpp-dependent. Viewed from the perspective of mechanism, results from our studies point to a role for Mmy in shaping and constraining the epidermal Dpp gradient.

Discussion

In the current report, we provide significant new insights into the mechanisms by which morphogen activity domains are spatially constrained in development. Although secreted Dpp/BMP is a potent and largely unconstrained morphogen in most developmental contexts, in the Drosophila embryonic epidermis *dpp*/Dpp expression and function are precisely regulated in time and space. Results from studies reported here and elsewhere reveal that at least two tiers of signaling antagonism contribute to this spatio-temporal restriction. In particular, we show that JNK/AP-1 and Dpp regulatory machineries function independently to limit bioactive Dpp signaling fields in the Drosophila embryonic epidermis. Whereas widespread transcriptional activation of epidermal *dpp* is suppressed by Raw, long-range Dpp signaling is suppressed by Mmy. Thus, Mmy and Raw, despite their striking shared loss-of-function phenotypes, independently constrain the amplitude and range of Dpp activity during dorsal closure. Moreover, the Mmy and Raw pathways are not redundant, as loss of either leads to ectopic Dpp activity.

mmy, which encodes the Drosophila UDP-N-acetylglucosamine pyrophosphorylase, represents the fourth and newest member of the *raw*-group of dorsal-open mutants. As we showed previously for the three defining members of this group (*raw*, *puc*, and *rib*; Bates et al., 2008), *mmy* mutant's exhibit: (1) defects in two Dpp-dependent embryonic processes – dorsal closure and ventral cuticle differentiation, as well as (2) expansion of epidermal Dpp activity. *mmy* is, however, distinct from the two members of the *raw*-group whose modes of action have been defined previously. Specifically, we have demonstrated that unlike the novel raw protein and the Puckered MKP, which mediate their effects on Dpp via their modulation of a JNK/AP-1 signaling cascade, Mmy mediates restriction of Dpp activity directly. Moreover, our data provide strong evidence that a target of the *mmy*-encoded UDP-N-acetylglucosamine pyrophosphorylase functions as a developmental switch in the Drosophila epidermis, eliminating long-range Dpp signaling.

Modulation of Dpp signaling activity by Mmy and UDP-GlcNAc

Dpp signaling has been exceptionally well characterized in patterning Drosophila embryos and imaginal discs (O'Connor et al., 2006). A key feature of Dpp action in these two developmental contexts is that Dpp exerts its effects on immediate neighbors, as well as on more distant cells, via an extracellular morphogen gradient. Based on these clear and strong precedents, it has been widely assumed that during dorsal closure Dpp is secreted from its LE cell source and subsequently functions non-cell autonomously to direct cell changes in the lateral epidermis that are essential for Drosophila morphogenesis during dorsal closure (Fernandez et al., 2007). Evidence from studies presented here and elsewhere (Wang et al., 2008) have, however, led us to a new model of Dpp function in LE cells during closure. As discussed below, it is now apparent that during dorsal closure LE Dpp does not function exclusively as a long-range epidermal signal. Moreover, long-range epidermal Dpp signaling is constrained during dorsal closure, and the *mmy* gene product plays a central role in refining this vital epidermal Dpp activity profile.

Our studies have their foundation in the observation that embryos homozygous for several *mmy* alleles (mmy^{1}, mmy^{LM1}, mmy^{LM16}, mmy^{LM24}) suffer a fully penetrant embryonic lethality associated with a cuticle phenotype that we have shown here and elsewhere (Byars et al., 1999) is due to misregulated Dpp signaling – spatially, temporally, and quantitatively. Moreover, our data lead us to suggest that Dpp secreted from LE epidermal cells encounters an as yet unidentified sugar-modified sink. For example, Mmy-dependent modification of either an ECM or Dpp receptor component might constrain Dpp activity (by either degradation or titration). In contrast, we suspect that in the absence of *mmy*, Dpp sequestration is down-regulated and Dpp is consequently free to: (1) move away from its source, and (2) generate the expanded and more robust signaling field that we visualize in epidermal *dpp*/Dpp expression and activity profiles (Fig. 6).

Somewhat contrary to the established paradigm of Glc-NAc/glypican function in modulating Dpp signaling in the wing disc of Drosophila, we found that Mmy limits rather than augments the Dpp signaling field in the embryonic epidermis. There is precedence for opposing effects of glypicans in Hedgehog signaling, where the sugar modification has been shown to augment signaling in Drosophila while down-regulating signaling in mouse (Beckett et al., 2008). In addition, and although not previously recognized as such, our current studies, along with previous studies focusing on LE *dpp* transcription (Arora et al., 1995; Johnson et al., 2003; Letsou et al., 1995), indicate that the embryonic epidermis is competent to regulate *dpp* in an auto-regulatory fashion as it is in several other developmental contexts (Capovilla et al., 1994; Hursh et al., 1993; Panganiban et al., 1990; Staehling-Hampton and Hoffmann, 1994). Thus, epidermal signaling abnormalities in *mmy* mutants can be amplified via a feed forward mechanism of intercellular communication. Moreover, in this context at least, cytokine diffusion represents the default state. Finally, it is notable that while pMAD activates *dpp* transcription beyond the leading edge in *mmy* mutant embryos, the more modest amounts of pMAD visualized in dorsal-closure stage wild-type embryos are insufficient to activate *dpp* transcription in these same cells. Quantitation of this difference in wild-type and *mmy* mutant embryos defined a three-fold threshold for *dpp*-activation by pMAD.

Future experiments will require identification of the Mmy-dependent glycosylated protein product(s) essential for Dpp restriction to LE cells. Certainly there are several potential candidates; very high among these are Dpp receptors (and co-receptors). Both Tkv and Punt, the type I and type II receptors functioning in dorsal closure, harbor multiple potential glycosylation sites; modification of any of these might enhance ligand affinity for its receptor. The type I Dpp receptor Tkv has been shown previously to be a means for Dpp signal down-regulation (Lecuit and Cohen, 1998), and the type II Dpp receptor has been

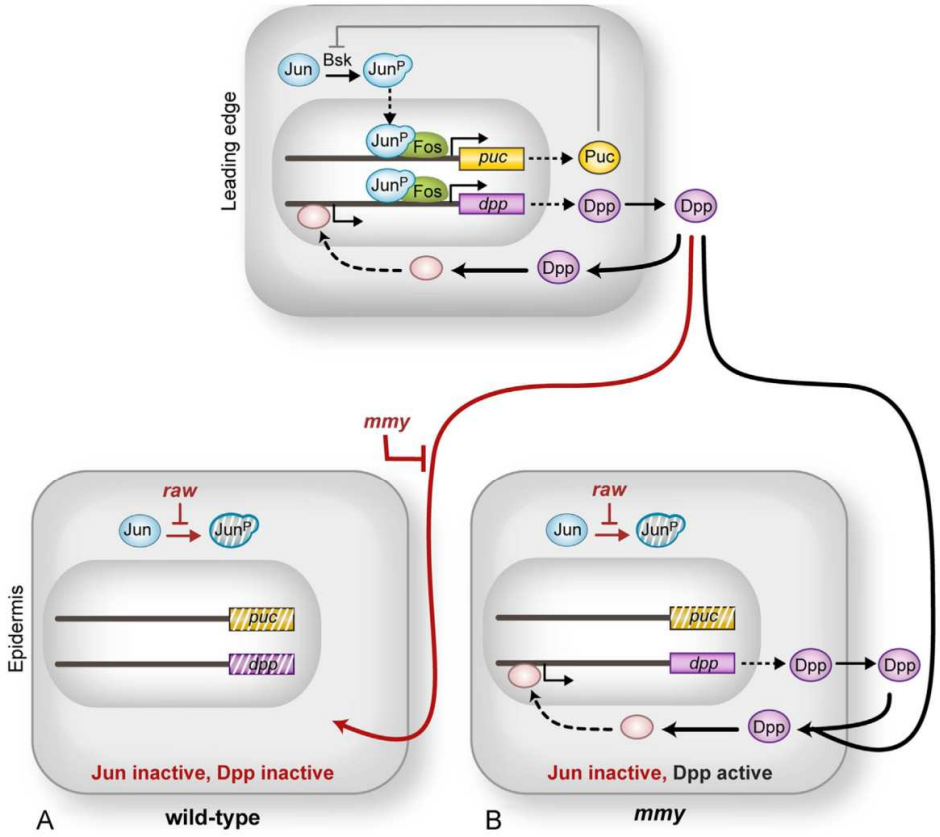

Fig. 6. Modeling LE dpp restriction. JNK/AP-1 and Dpp signaling in LE cells is shown at top. Dorsolaterally-positioned epidermal cells are shown below: (A) In wild-type cells, *raw* prevents accumulation of the bioactive, phosphorylated form of Jun in the dorsolateral epidermis, while *mmy* prevents Dpp from signaling between LE and neighboring epidermal cells. (B) In *mmy* mutants, LE Dpp activates signaling in neighboring epidermal cells, where autoregulatory *dpp* feeds forward to activate its own transcription.

shown previously to be a dosage sensitive component of the signaling pathway (Simin et al., 1998). Collagen might also be modified by GlcNAc as it has been suggested that the Drosophila collagen Viking sequesters and limits the Dpp signaling range in the germarium (Wang et al., 2008). The Dally or Dally-like glypicans, although augmenting Dpp diffusion rates at previously defined sites of Dpp action in Drosophila and also play limiting roles in restricting the movement of a morphogen signal in other organisms (Gumienny et al., 2007), are not likely to function downstream of Mmy in regulating epidermal signal transduction. Loss-of-function *dally* or *dally-like* mutations are associated with neither dorsal closure defects nor embryonic lethality more generally. In addition, *dally* and *dally-like* double mutant studies with *mmy* show no genetic interactions (GH, unpublished). Finally, it is possible that Mmy contributes to the modification of intracellular proteins or chromatin through O-linked glycosylation. We do not favor this mechanism of action as the effect that we see is dependent upon leading edge *dpp* expression, and this strongly implicates signaling itself as the Mmy-dependent step in Dpp regulation. Moreover, as for *dally* and *dally-like*, loss of function mutations of the single O-linked transferase in Drosophila are not associated with defects in dorsal closure (Ingham, 1984).

As a final point, Mmy's role as a Dpp signaling antagonist likely extends beyond the embryonic epidermis and dorsal closure, as Schimmelpfeng and coworkers have reported ectopic Dpp activity in a mmy^7 background in the Drosophila eye (Schimmelpfeng et al., 2006). The failure in this study to detect ectopic *dpp* in the epidermis of mutants likely reflects the low penetrance of dorsal closure defects in the mmy^7 background. We too are unable to detect ectopic *dpp* in alleles with low penetrance of dorsal closure defects. For *raw* mutants as well, there is a gradient of *dpp* expansion that parallels the strength of molecularly characterized loss-of-function alleles (Bates et al., 2008). Furthermore, this result highlights the fact, that in terms of signaling, less gylcosyolation does not necessarily correlate with more signaling; i.e. specific thresholds of decreased gylcosylation/modifications might either augment or limit signaling.

Conclusions

During development, a surprisingly small number of signaling cascades are used again and again to mediate communication within and between cells, and to regulate a variety of cellular responses, including proliferation, differentiation, survival, and death. Among these essential signaling pathways are two (Dpp and JNK) that we have studied here in the context of Drosophila dorsal closure, and for which conserved functions have been repeatedly demonstrated. In virtually all animal models, the Dpp (TGF-β/BMP) pathways mediate both short- and long-range intercellular communication in response to the eponymous, diffusible extracellular cytokine. Similarly, the conserved JNK (MAPK)

pathways activate transcription of gene suites in virtually all animal models in response to a variety of both extracellular and intracellular stimuli, including peptide growth factors, cytokines, and hormones, as well as diverse cellular stressors including oxidative and endoplasmic reticulum stress. Deviation from the strict control of any of these signaling pathways has been implicated in the development of countless human developmental abnormalities, degenerative diseases, and cancer pathologies. The fact that health and development consequences of misregulated signaling are so far-reaching has prompted numerous research programs to seek a better understanding of how these complex regulatory circuits are controlled - at the levels of both activation and repression.

Approaching this problem by dissecting signaling circuitry in the model genetic system of Drosophila dorsal closure, we have made considerable progress in unraveling the complex circuitry that links JNK to Dpp, and both to epithelial morphogenesis. Our previous studies of *raw* have revealed its role as a master regulator in the complex circuitry of the developing Drosophila embryo. Our current studies of *mmy* reveal that UDP-N-acetylglucosamine pyrophosphorylase activity is required to spatially limit Dpp activity in a JNK/AP-1-independent fashion. Together, our studies of the Drosophila Mmy and raw signaling antagonists lead us to a fuller understanding of the molecular mechanisms governing coordinated signaling pathways, which throughout the animal kingdom control a variety of biologically essential cell growth, proliferation, and differentiation pathways.

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CHAPTER 3

CHONDROITIN-MEDIATED MODULATION OF DPP SIGNALING IN THE EMBRYO

Abstract

Mummy (Mmy), a UDP-N-acetylglucosamine pyrophosphorylase that synthesizes UDP-GlcNAc, functions in the epidermis to limit Dpp signaling, but the precise mechanism of signal antagonism remains unclear. Mmy mutants have reduced protein glycosylation, so we hypothesized that a glycosylated protein is the likely antagonist. To identify the glycosyl-modified factor(s) functioning downstream of *mmy* to enact Dpp signal antagonism, we examined the loss-of-function cuticle phenotypes of 23 of the 25 Drosophila β-1,3 glycosyltransferases. From this screen we identified *wanderlust* (*wand*), a gene with homology to the evolutionarilyconserved *Chondroitin sulfate synthase 2*, and its upstream partner *UDP-galactose 4'-epimerase* (*Gale*) as antagonists of Dpp. *Gale* and *wand* are expressed in the mesoderm underlying the Dpp-secreting leading edge cells of the epidermis. We observed that *wand* was required not just for epidermal signal antagonism, but also for proper mesodermal signaling and dorsal vessel development. Results from our studies point to a role for chondroitin sulfate in converting a long-range signaling molecule into a short-range one and provide evidence for the first-ever localized signaling sink functioning *in vivo*.

Introduction

During embryonic development, numerous embryonic cells differentiate, migrate, and divide in coordinated, repeatable patterns. One mechanism behind this reproducible execution is morphogen signaling. Morphogens were first defined by Alan Turing in his landmark paper, "The Chemical Basis of Morphogenesis," as diffusible chemicals that can self-organize to form a

signaling gradient, wherein differential signal concentrations determine cellular reaction (Reviewed in Rogers and Schier, 2011; Turing, 1952). In 1969, Lewis Wolpert refined our understanding of morphogen gradients with the French Flag model, wherein a diffusible ligand forms a gradient between a source and distant cells, and cells produce a response that is dictated by the local concentration of morphogen the cell perceives (Wolpert, 1969). Shortly afterwards, Francis Crick realized that signal production and spreading will eventually lead to an even distribution of signal across all cells and noted that generation of a stable continuous gradient requires a "sink," a signaling component located at a distance from the source that destroys the signal (Crick, 1970). Morphogen gradients could most easily be explained by rapid, free diffusion in a closed system with constant destruction of the signaling ligand by a signaling sink. In time the model would be refined, in that in order to form a stable gradient, a sink need not be localized, and the effect of ligands binding to their receptors could in fact be sufficient to form a stable signaling gradient (Yu et al., 2009); overexpression studies indicated that receptors can serve as a sink for their ligands, as indicated by decreased signaling of Drosophila BMP homologue Decapentaplegic (Dpp) in cells adjacent to where the receptor Thickveins (Tkv) is ectopically increased (Lander et al., 2009; Mizutani et al., 2005). Additionally, not all morphogens establish gradients through the classical source-sink mechanism; the shape of the Bicoid gradient is primarily established through mRNA diffusion and distribution (Lipshitz, 2009; Spirov et al., 2009).

Despite the fact that a localized sink, either near or distant from the signaling source, has never been successfully identified in vivo, there is compelling evidence that one might exist. Several molecules have been shown to have the ability to bind to extracellular ligands and in some cases remove them from the system. With respect to BMP/Dpp signaling, there is evidence that the proteoglycan Perlecan serves as a sink for growth factors in the mammalian growth plate (Aviezer et al., 1994; Deguchi et al., 2002; Mongiat et al., 2000; Smith et al., 2007). In Drosophila, artificial BMP/Dpp sinks can be created through overexpression of heparan sulfate (HS) proteoglycans Dally and Dally-like (Belenkaya et al., 2004b) or overexpression of signaling receptors (Lander et al., 2009; Mizutani et al., 2005). In Drosophila Decapentaplegic (Dpp) signaling, for instance, a localized sink could be functioning to restrict Dpp to the hub cells of

Drosophila adult testes and ovaries, as well as the leading edge of the mesoderm and epidermis during embryonic dorsal closure (Humphreys et al., 2013; Kawase et al., 2004; Shivdasani and Ingham, 2003; Song et al., 2004; Yang and Su, 2011)

In addition to shaping signaling gradients so that positional information can be interpreted, signaling sinks may serve additional vital functions. As signaling morphogens control cell growth and differentiation, their misregulation is not surprisingly associated with diseases such as cancer and developmental abnormalities such as fibrodysplasia ossificans progressiva (Alarmo et al., 2006; Fan et al., 1997; Gobbi et al., 2002; Jin et al., 2001; Kaplan et al., 2006; Katoh and Terada, 1996; Li et al., 2010; Nishisho et al., 1991; Polakis, 2012; Singh and Morris, 2010). Often the tumorigenic properties of the cancer are reduced when signaling imbalance is rectified. Indeed, in order for survival, signals must often be restricted to the cells that require it and kept from those that do not, and signaling sinks may serve a vital role in signal antagonism. In the work presented here, I focus on how signaling sinks are utilized to antagonize BMP/Dpp signaling in the embryonic development of Drosophila.

BMPs are an evolutionarily important group of signaling molecules conserved in vertebrates and invertebrates, with evidence suggesting a common ancestor at least 600 million years ago (Padgett et al., 1993). BMPs were first identified and named for their ability to induce bone development (Urist, 1965). Since their discovery, BMPs have been shown to play roles in diverse developmental and morphological processes, including organogenesis (Frasch, 1995; Lyons et al., 1990; Mandel et al., 2010; Winnier et al., 1995), wound healing, stem cell maintenance (Kawase et al., 2004; Shivdasani and Ingham, 2003; Song et al., 2004; Ying et al., 2003), and others.

BMPs are diffusible signaling ligands that form signaling gradients within and between tissues and signal through binding to a dimeric receptor complex containing a type II and a type I subunit; varying the components of the receptor affects specificity to specific BMP ligands. When the ligand-receptor complex is formed, the type I receptor subunit becomes phosphorylated and active. Phosphorylated type I receptor will then recruit and phosphorylate a Smad signal transducer, which subsequently enters the nucleus and binds to BMP-responsive targets to

activate and enhance transcription (Massagué, 1998).

The fruit fly *Drosophila melanogaster* has been historically useful in studying BMP signaling. Many of the evolutionarily conserved proteins involved in activating BMP signaling have been identified in Drosophila, due to the recognition of shared loss-of-function cuticle defects of signaling components mutated in the Heidelberg screen for mutants affecting the embryonic pattern of larval cuticle (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984). The Drosophila epidermis is particularly useful in the study of BMP signaling, as this is where zygotically encoded Drosophila BMP homologue *decapentaplegic* (*dpp*) is first required, and *dpp* signaling mutants (with the exception of the *dpp* mutant itself, which has a ventralized phenotype) have well-characterized and easily identifiable dorsal closure defects (Andrew et al., 1997; Brummel et al., 1994; Letsou et al., 1995). Dorsal closure is an essential early embryonic process during which the laterally positioned epidermal sheets extend to the dorsal midline where they fuse to enclose the entire embryo in epidermis. This extension is a nonmitotic process driven by changes in cell shape. Dorsal closure and similar morphogenetic closure processes in higher eukaryotes are initiated and coordinated through conserved, sequentially activated Jun-N-terminal kinase (JNK)/AP-1 and Dpp signaling pathways (reviewed in Xia and Karin, 2004).

Dorsal closure initiates with restricted activation of JNK/AP-1 and *dpp* expression in the leading edge (LE), the dorsal-most cells of the lateral epidermis sheet. Tight control of JNK/AP-1 signaling is vital; loss of positive or negative pathway regulators leads to a failure of the epidermis to spread dorsally, resulting in incomplete closure. The success or failure of dorsal closure and other embryonic body patterning processes can be evaluated by examining the larval cuticle, an exoskeletal layer of chitin and protein secreted by the *Drosophila* epidermis in a late step of embryogenesis. Loss of function of Drosophila *dpp* antagonists results in a specific cuticle phenotype characterized by hypotrophy of ventral denticle belts and a dorsal hole or pucker due to improper dorsal closure. This phenotype is shared by all Drosophila *dpp* antagonists studied to date, with the exception of *brinker* (*brk*), a competitor for binding to Dpp response elements functioning more laterally in the epidermis. *brk* mutants only exhibit hypotrophy of the ventral denticle belts, with none of the closure defects (Jaźwińska et al., 1999). This shared loss of

function cuticle phenotype has been successfully used to identify other antagonists of *dpp,* including *raw*, *ribbon*, *puckered*, and *mmy* (Byars et al., 1999; Humphreys et al., 2013; Jack and Myette, 1997; Ring and Martinez Arias, 1993).

mmy is distinct from several other dpp antagonists in that its effects are not mediated through action of JNK/AP-1 signaling, but are directly on *dpp* (Humphreys et al., 2013). *mmy* encodes the single *Drosophila* UDP-N-acetylglucosamine pyrophosphorylase, an enzyme catalyzing the final step of UDP-GlcNAc biosynthesis (Araújo et al., 2005). Certain *mmy* hypomorphs, such as allele mmy^{1}, exhibit defects in dorsal closure yet have intact chitin, signifying that the mmy^{1} dorsal closure defect stems from an alternative requirement for UDP-GlcNAc (Humphreys et al., 2013; Tonning et al., 2006). Aside from chitin synthesis, UDP-GlcNAc is utilized for a variety of functions including N- and O-linked glycosylation as well as synthesis of glycosyl-phospatidylinostiol, HS, and chondroitin sulfate (CS) (Breitling and Aebi, 2013; Hardingham and Fosang, 1992; Low, 1989; Wells et al., 2001).

There are many diverse ways that UDP-GlcNAc could be utilized downstream of mmy to enact Dpp antagonism, and it is the focus of the study presented here to identify the precise mechanisms and molecules antagonizing *dpp* through UDP-GlcNAc utilization. In this study we show that loss of function of *CG43313*, which we have named *wanderlust* (*wand*), suggests a role for the CS biosynthetic pathway in Dpp signaling antagonism. As proteins have already been implicated in promoting and antagonizing extracellular signaling, we chose to first focus on proteins as the likely antagonist(s). *wand* encodes a putative chondroitin sulfate synthase, and its restricted expression in embryonic cardiac cells suggests it may function to synthesize a CS sink to antagonize Dpp signaling in the epidermis.

Materials and methods

Drosophila strains

Fly lines used for this study include w^{1118}, mmy^{1}, mmy^{P15133}, *Tub-Gal4* (Bloomington), $mmy^{l(2)03851}$ (M. Krasnow), $Gale^{F00624.4}$ and $Gale^{\Delta y}$ (J. Fridovich-Kiel), sdc^{23} and sdc^{97} (G. Vorbrüggen), *B2-3-20* (E. Bier), $CG43313^{PL61}$ and $CG43313^{PL69}$ (A. Vincent), and the Vienna

Drosophila UAS-shRNA lines (Dietzl et al., 2007) v2598, v2601, v2826, v5027, v6176, v6177, v7262, v7263, v7394, v7427, v7949, v8107, v12079, v13474, v16981, v16982, v21761, v26517, v29084, v29085, v33366, v33368, v35572, v35573, v42781, v44939, v45194, v45457, v46419, v46421, v51977, v100016, v100185, v101307, v101417, v101660, v102288, v104256, v104281, v105791, v106134, v106605, v106839, and v107840 (VDRC).

Glycosyltransferase screen

To identify glycosyltransferases functioning downstream of *mmy* to enact *dpp* antagonism, we mated Tub-GAL4 virgins to UAS-shRNA males from glycosyltransferase RNAi lines to induce early, ubiquitous depletion of maternal and zygotic transferase mRNA. We then compared larval cuticles (described below) to *mmy* mutant larval cuticles to test for shared loss-of-function cuticle defects. The screen included all 25 predicted Drosophila β1,3-glycosyltransferases (Correia et al., 2003).

Cuticle analyses

For embryonic cuticle analysis, animals were dechorionated in 50% bleach solution and incubated overnight at 60℃ in One-Step Mounting Medium (30% CMCP-10, 13% lactic acid, 57% glacial acetic acid). In some cases embryos were devitellined prior to One-Step Mounting Medium incubation by shaking for 1 minute in equal parts methanol and heptane. Cuticles were visualized with dark field microscopy.

Larvae dissections

To prepare larvae for fixing and staining, 3rd instar wandering w^{1118} larvae were placed in a drop of ice-cold PBS and bisected at the midpoint of the anterior/posterior axis. The anterior half was retained and inverted to expose internal organs and tissues to the solution. Inverted larvae were fixed, washed, hybridized with an digoxigenin-labeled mRNA probe, as described previously (Byars et al., 1999). Following staining, individual tissues and organs were dissected and visualized by DIC optics.

RNA *in situ* hybridization

For *in situ* hybridization, we generated digoxigenin-labeled probes as described previously (Byars et al., 1999). Probes were detected with anti-digoxigenin-AP Fab fragments (Roche) and visualized with DIC optics.

Immunolocalization

Alkaline phosphatase immunolocalization studies were performed as described (Sullivan et al., 2000). Fluorescent Phospho-Mad visualization was performed as previously described (Humphreys et al., 2013) using confocal techniques. For immunolocalization studies, we used rabbit anti-Phospho-Smad1,5 Ser463/465 (1:20, Cell Signaling Technology), mouse anti-β-Gal (1:500, Promega), goat anti-mouse alkaline phosphatase (1:2000, Promega), goat anti-rabbit alkaline phosphatase (1:2000) (Jackson ImmunoResearch), and goat anti-rabbit Alexa Fluor 488 antibodies (1:200, Invitrogen Molecular Probes).

Western Blotting

For western blotting studies, control and experimental protein lysates were made from embryos 5–17 hours after egg lay (AEL). Absence of a GFP-marked balancer chromosome was used to distinguish mutant homozygote embryos from wild-type siblings. Protein lysates were separated on SDS-acrylamide gel and analyzed by western blotting using anti-Dally-like protein antibody (1:1000, Developmental Studies Hybridoma Bank) and mouse anti-Tubulin control (1:50000, Amersham). HRP-conjugated donkey anti-mouse was used as the secondary antibody (Jackson ImmunoResearch). Blots were stripped using a mild stripping protocol (Abcam) prior to being reprobed for Tubulin control.

Results

There are a number of glycosyltransferases that function downstream of Mmy to carry out glycosyl-modifications. We hypothesized that loss of function of a transferase that glycosylates a protein involved in Dpp antagonism would reproduce the scoreable *mmy* mutant cuticle

phenotype. To identify the protein(s) modified downstream of Mmy to enact Dpp antagonism, we performed an RNAi screen against 23 of the 25 predicted Drosophila β-1,3 glycosyltransferases (Correia et al., 2003) and screened the embryos for the cuticle defect exhibited in *mmy*^{1} mutant embryos (Table 3.1) (Humphreys et al., 2013; Nüsslein-Volhard et al., 1984). Loss of function of a single glycosyltransferase, encoded by *CG43313*, exhibited a cuticle defect similar to that seen in *mmy*^{1} mutants (Figures 3.1C and 3.1E), suggesting that this transferase modifies a protein involved in Dpp antagonism. This *CG43313*-encoded transferase, which we have named Wanderlust (Wand), is homologous to human Chondroitin sulfate synthase 2 (Yada et al., 2003a) (Figure 3.2) and is predicted to function in utilizing UDP-GalNAc to synthesize chondroitin.

Next we tested if *mmy* hypomorphic alleles have sufficient loss of UDP-GlcNAc synthesis to result in glycosylation defects; for this purpose, we used western blot analysis to analyze shifts in molecular weight of Dally-like protein (Dlp), a HS proteoglycan consisting of a protein core of 85 kDa weight and attached HS chains of variable lengths (Figure 3.3). In wild-type embryonic lysates, HS-modified Dlp appears as a broad band in the 130–160 kDa range of the blot, whereas in two independently generated *mmy* hypomorphs the Dlp band shifted to average 100 kDa; these data suggest truncation in HS chain length in *mmy* mutants and confirm that hypomorphic alleles of *mmy* exhibiting Dpp signaling abnormalities have insufficient UDP-GlcNAc synthesis in order to carry out full glycosylation. Additionally, we noted that the intensity of the Dlp band was decreased in *mmy* mutant embryos, though whether this Dlp decrease is due to a decrease in translation, an increase in degradation, or a lesser affinity of the anti-Dlp antibody for Dlp with truncated HS chains is unknown.

There are two lethal alleles of *wand* (*CG43313*^{PL61} and *CG43313*^{PL69}) that were generated by P-element insertion into the gene region (Bourbon et al., 2002). We tested these alleles for phenotypes characteristic of ectopic *dpp* expression, but as mutant animals die in the larval stage, they did not reproduce the *mmy* cuticle phenotype, nor did they have expanded *dpp* transcription (data not shown). This may be due to a high level of maternal deposition of *wand* or to a hypomorphic nature of the alleles (Figure 3.4D). Chondroitin synthesis lies downstream of Mmy in a linear biochemical pathway (Figure 3.1A). To confirm that the effects of *wand* RNAi are

Table 3.1 – β-1,3 glycosyltransferase genes screened for *mmy*-like cuticle phenotypes			
Gene name	Transformant ID	Viability	Cuticle phenotype
C1GalTA	2826	semi-lethal	wt
CG8708	8107	Viable	-
	45194	viable	-
CG2975	2601	viable	-
CG3119	2598	viable	-
	105791	viable	-
twg	6176	viable	-
	6177	viable	-
CG34056	7427	viable	-
CG34057	21761	viable	-
CG18558	33366	semi-lethal	wt
	33368	viable	-
CG9220	29084	viable	-
	29085	viable	-
	106839	lethal	wt
β3GalTII	7949	viable	-
	106134	viable	-
CG9109	16981	lethal	misformed denticles, 79/212 embryos
	16982	lethal	no cuticle, 0/95 embryos
	100016	lethal	wt
brn	44939	lethal	wt
	45457	lethal	wt
CG11357	5027	viable	-
	108467	semi-lethal	wt
CG8673	100185	viable	-
	100185	viable	-
CG33145	7394	viable	-
	104256	lethal	wt
CG30037	12079	viable	-
	13474	viable	-
	101660	viable	-
CG30036	101307	viable	-
CG3038	35572	viable	-
	35573	viable	-
CG2983	7262	viable	-
	7263	viable	-
	104281	lethal	wt
wand	26519	semi-lethal	*mmy*-like, 35/60 cuticles
GlcAT-I	107840	lethal	wt
	46421	viable	-
GlcAT-S	42781	viable	-

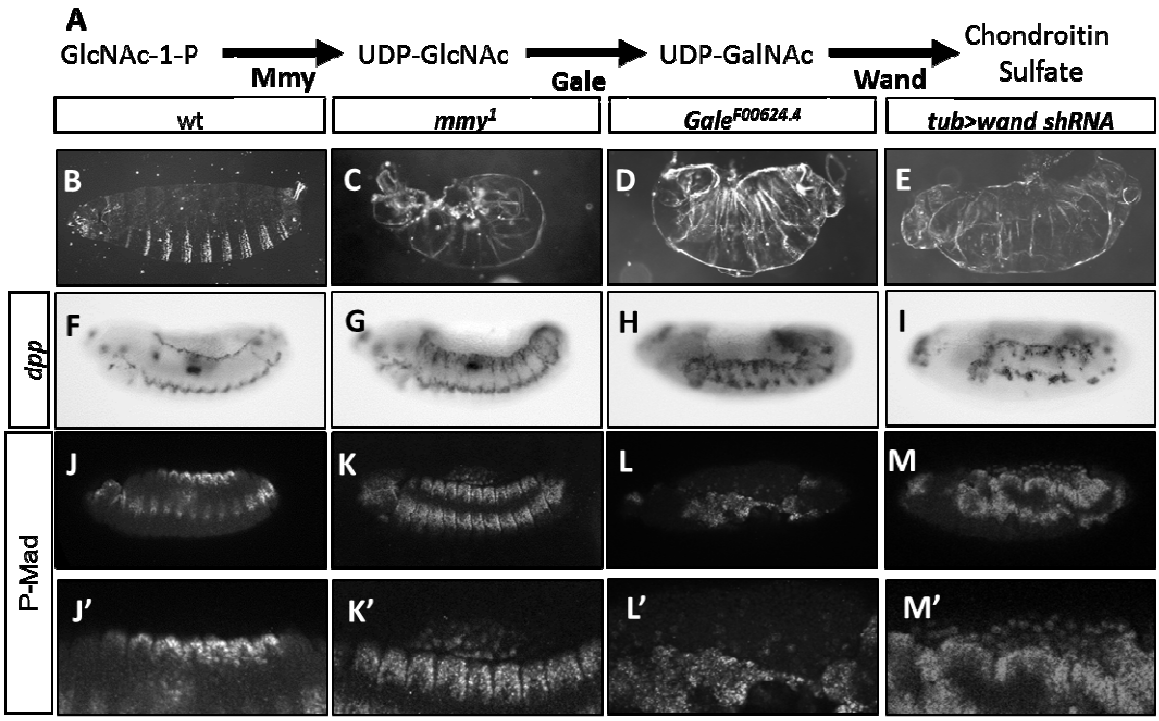

Figure 3.1. Chondroitin sulfate synthesis is required for Dpp antagonism. (A). Biochemical pathway for converting GlcNAc-1-P to chondroitin. Larval cuticles, *dpp* mRNA, and Mad phosphorylation in embryos with chondroitin sulfate synthesis defects. (B) A wild-type cuticle. (C) A mmy^{1} mutant cuticle has a dorsal pucker, germband retraction and head involution defects, and hypotrophy of the ventral denticle belts. This phenotypes is shared by loss-of-function of downstream genes (D) *Galactose epimerase (Gale),* which converts UDP-GlcNAc to UDP-GalNAc, and (E) *wand*, which encodes a putative chondroitin sulfate synthase. This shared cuticle phenotype is indicative of ectopic *dpp* in the epidermis. (F–I) *dpp in situ* in stage 13 embryos. (F) Lateral view of a wild-type embryo. *dpp* expression is restricted to the single row of leading edge epidermal cells during dorsal closure. Expansion of *dpp* transcription into the dorsolateral epidermis is observed in (G) mmy^{1}, (H) $Gale^{F00624.4}$, and (I) *CG43313* embryos. (J–M) Phospho-Mad in the epidermis of Drosophila embryos, single slices. (K) mmy^{1} and (L) $Gale^{F00624.4}$ mutants, and (M) *wand* RNAi embryos have an expansion of Dpp signaling activity (detected by Mad phosphorylation) in the epidermis beyond what is observed in (J) wild-type embryos.

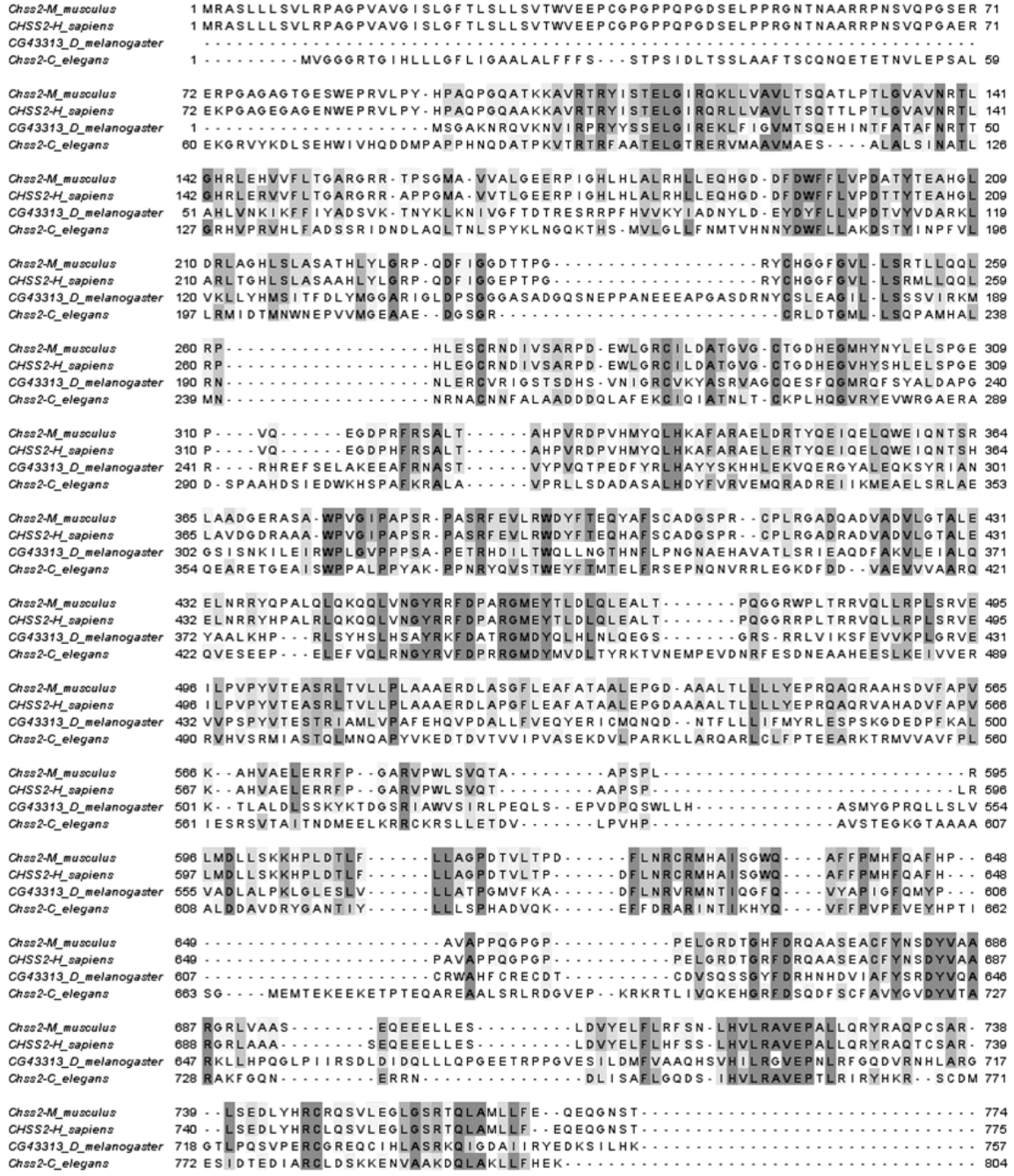

Figure 3.2. Wand is homologous to Chondroitin sulfate synthase 2. Multiple sequence alignment between Chondroitin sulfate synthase 2 (M. musculus), Chondroitin sulfate synthase 2 (H. sapiens), CG43313 (D. melanogaster), and Chondroitin sulfate synthase 2 (C. elegans). Sequences were aligned using ClustalX 2.1 (Larkin et al., 2007), and the alignment was shaded according to BLOSSUM62 scores using Jalview 2.8.1 (Waterhouse et al., 2009).

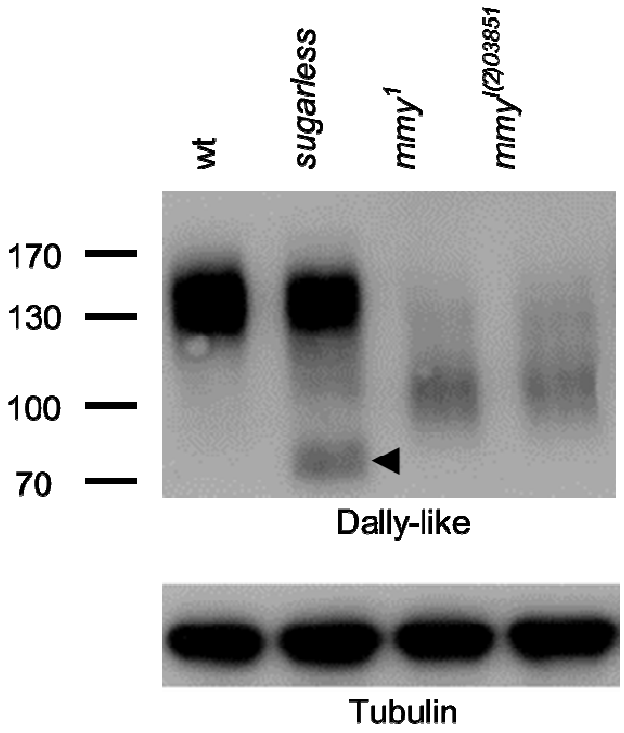

Figure 3.3. *mmy* hypomorphs have defective glycosylation. Western blot analysis of Dally-like protein (Dlp). Dlp consists of a core protein with molecular weight 85 kDa, which is post-translationally modified by glycosylation with heparan sulfate chains of variable length. The glycosylated protein appears as a smear of products of approximate molecular weight 130–160 kDa. The core protein is denoted by the black arrow in the *sugarless* lane, as *sugarless* is required to initiate heparan sulfate attachment; the presence of glycosylated Dlp is likely the product of enzyme translated from maternal mRNA. A reduction in Dlp molecular weight is observed in two independently generated hypomorphic *mmy* mutants, suggesting decreased availability of UDP-GlcNAc for posttranslational glycosylmodifications; there is also a generalized decrease of band intensity in *mmy* mutant lysates, suggesting a decrease of Dlp protein in these genetic backgrounds.

due to a loss of chondroitin, we turned to the gene directly upstream of *wand* in the chondroitin biosynthesis pathway. The Drosophila gene *UDP-galactose 4'-epimerase* (*Gale*) is required to convert UDP-GlcNAc into UDP-GalNAc (Sanders et al., 2010), the substrate of chondroitin sulfate synthases. Given this, genetic and biochemical models predict that *Gale* mutant embryos would have the same loss-of-function cuticle defects as *mmy* embryos. When tested, this prediction held true (Figure 3.1D); the shared cuticle phenotypes of three consecutive enzymes in the CS biosynthesis pathway (Figures 3.1B–3.1D) suggests that CS is an important protein modification

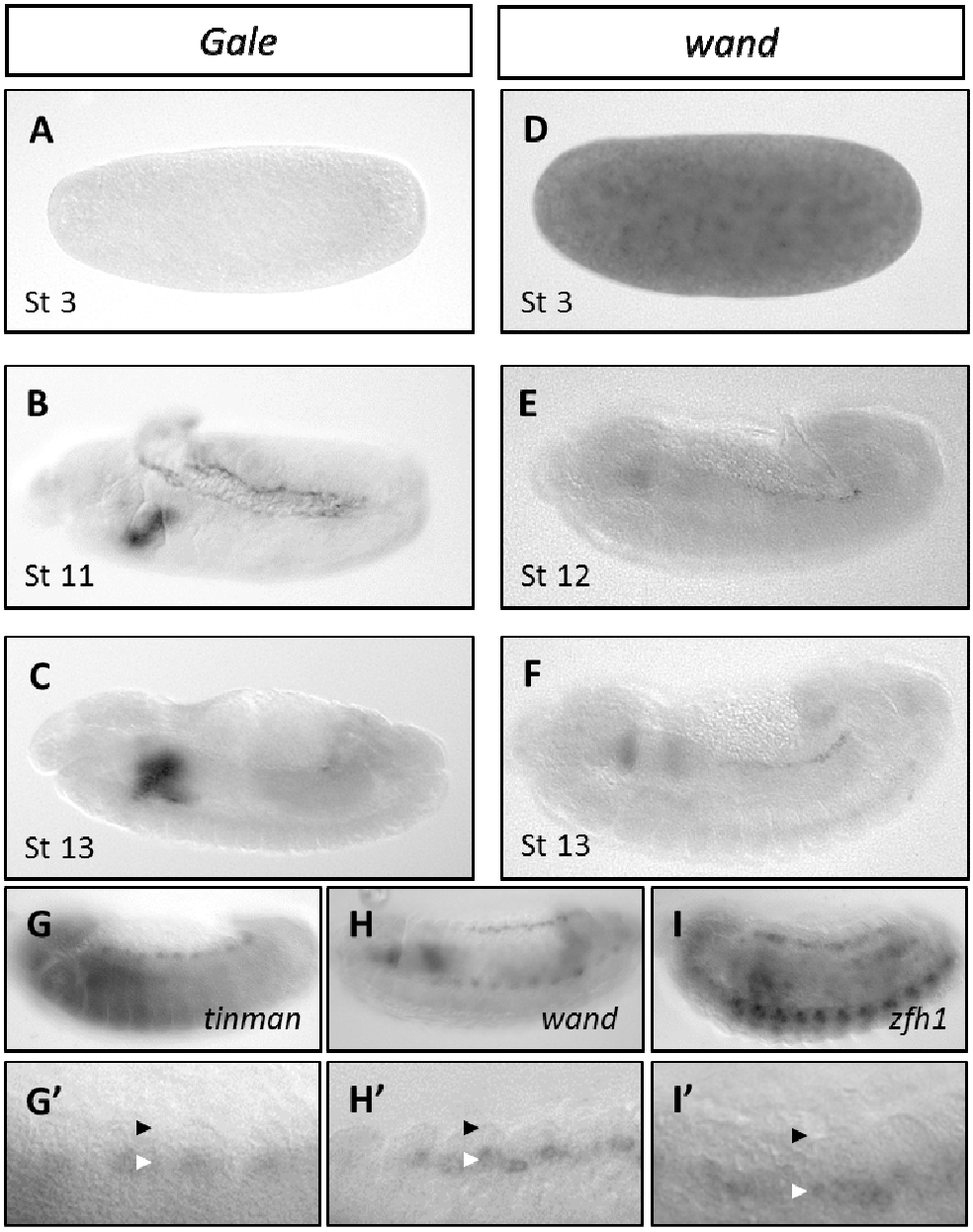

Figure 3.4. Embryonic expression patterns of *Gale* and *wand*. *Gale* mRNA (A) is not maternally deposited. (B) Expression behind the epidermal leading edge initiates during embryonic stage 11, (C) but diminishes by stage 13. *wand* mRNA (D) is maternally deposited. (E) Cardiac expression of *wand* initiates during stage 12 and continues through (F) stage 13 of embryogenesis. (G–I) A comparison of tissue expression of three transcripts, (G) *tinman,* a cardiac marker; (H) *wand*; and (I) *zfh1*, a pericardial cell marker. (G'–I') Black arrows denote the epidermal leading edge, whereas the white arrow depicts the location of mRNA transcript *in situ*. The gap distance between the two arrows is similar in *tinman* and *wand in situs*, suggesting that *wand* is expressed in cardiac cells, and not pericardial cells.

enacting Dpp antagonism.

To further characterize the roles of these genes in *dpp* antagonism, we examined the expression of *dpp* transcript in *Gale* mutant and *wand* RNAi embryos. In analysis of *dpp* transcript *in situ*, we noted that *Gale* mutant and *wand* RNAi embryos, like *mmy*^{1} mutant embryos, exhibit ectopic *dpp* expression in the dorsolateral epidermis (Figures 3.1F–3.1I). Furthermore, *wand* RNAi embryos exhibit ectopic *dpp* expression overlying the peripheral nervous system and in the head fold, suggesting a general role in Dpp antagonism that is not restricted solely to the leading edge of the epidermis (Figure 3.1I).

To test if ectopic *dpp* expression is associated with a corresponding expansion of Dpp signaling fields, we probed Mad phosphorylation (P-Mad) in embryos, as P-Mad is the transducer of the Dpp signal (Hoodless et al., 1996). We observed that P-Mad-positive Dpp signaling fields were expanded in *Gale* mutants and wand RNAi embryos, in a similar manner of expansion found in *mmy* mutants (Figures 3.1J–3.1M).

We next examined the expression of *Gale* and *wand* to determine if their localization might suggest a mechanism of signal antagonism. To test if *Gale* and *wand* are expressed in a time and location consistent with a function in dorsal closure, we examined the embryonic localization of said mRNA transcripts *in situ*. We noted that *Gale* is not maternally deposited (Figure 3.4A), but is expressed near the LE epidermis during germband extension (Figure 3.4B); the quantities and timing of *Gale* and *wand* expression are consistent with measures reported in the modENCODE database (Graveley et al., 2011). During dorsal closure, *Gale* is absent from the leading edge, though staining in the salivary glands is prominent (Figure 3.4C). *wand* is maternally deposited in the early syncytial blastoderm, but zygotic expression is first noted in a similar pattern to *Gale* expression during germband extension and continuing in expression near the leading edge through dorsal closure. A closer comparison of *wand* expression and expression of the cardiac enhancer *B2-3-20* suggested that it is expressed in the cardiac cells (data not shown), although we could not rule out the possibility that *wand* is instead restricted to pericardial cells. To distinguish between these two potential locations of expression, we compared *wand* expression to expression of *zfh1*, a pericardial cell marker, and *tinman*, a cardiac

marker (Figures 3.4G–3.4I) (Lockwood and Bodmer, 2002; Su et al., 1999). *wand* and *tinman* are similarly expressed, placing wand expression in the cardiac cells of the developing Drosophila heart, a tissue that in fact contacts the epidermal leading edge and requires Dpp for its specification (Frasch, 1995). To further characterize *wand* and identify other areas where CS might play a role in signal regulation, we examined mRNA localization of *wand* in Drosophila larvae. We observed that *wand* is absent from most larval tissues (data not shown). However, there is mRNA expression in all imaginal discs, as well as in the gastric caeca (Figures 3.5A and 3.5B).

As *wand* and *tinman* are similarly expressed in the cardiac cells, we next tested if they were under similar transcriptional regulation. *tinman* expression in the mesoderm is dictated by Dpp signaling and under indirect control of upstream JNK/AP-1 in the leading edge epidermis (Frasch, 1995; Xu et al., 1998; Yin and Frasch, 1998). In order to test if *wand* expression was similarly under control of JNK/AP-1 or Dpp signaling, I probed *wand* expression in wild-type, Jra^{IA109}, mmy^{1}, and raw^{JG} mutant embyros. Tinman expression is expanded in the mesoderm in raw^{JG} mutants, whereas *tinman* mRNA is absent or reduced in cardiac cells in Jra^{IA109} mutants (Lockwood and Bodmer, 2002; Yang and Su, 2011). We noted that *wand* expression is maintained in the cardiac cells in all genetic backgrounds tested (Figures 3.5C–3.5E), indicating that expression of *wand* is independent of activity of JNK/AP-1 and Dpp.

The cardiac-restricted expression pattern of *wand* is consistent with the localized expression of CS proteoglycans Syndecan (Sdc), Terribly reduced optic lobes (Trol), Kon-tiki, and Multiplexin, which are expressed in embryonic cardiac cells (Friedrich et al., 2000; Hammonds et al., 2013; Knox et al., 2011; Medioni et al., 2008; Momota et al., 2011; Tomancak et al., 2007). This expression pattern suggests that the *wand*-dependent mechanism to antagonize epidermal Dpp in fact originates in the mesoderm, and direct contact with the Dpp source suggests a potential model for a CS proteoglycan manufactured in the cardiac cells to act as a localized Dpp sink to prevent Dpp reaching sufficient thresholds in the epidermis to drive Dpp autoregulation (Figure 3.6) (Crick, 1970; Humphreys et al., 2013; Johnson et al., 2003). When the sink is lost due to loss of function of *mmy*, *Gale*, or *wand*, Dpp appears to reach the dorsolateral epidermis in

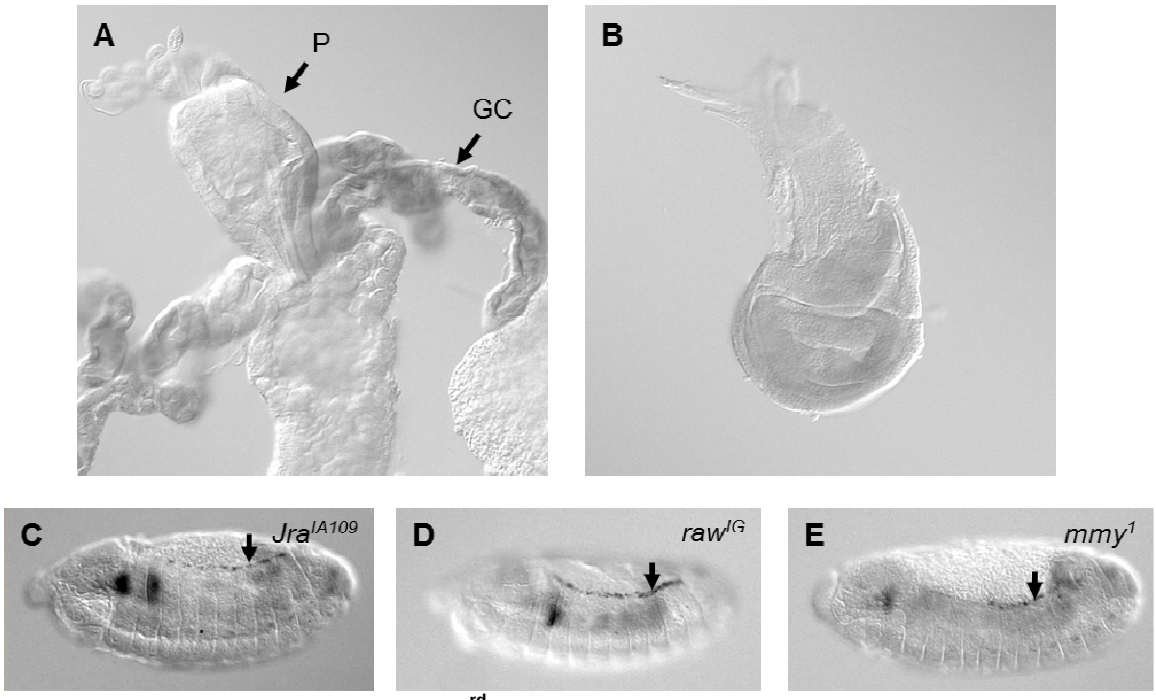

Figure 3.5. Expression of *wand* in 3rd instar larvae and transcriptional control of *wand* in embryos. (A) *wand* mRNA is expressed in the gastric cecae (GC), but is absent from the rest of the foregut and midgut, including the proventriculus (P). (B) wand mRNA is also present in imaginal discs, such as this wing disc. *wand* remains restricted to the cardiac cells (and its expression is not ablated) in (C) Jra^{IA109}, (D) raw^{JG}, and (E) mmy^{1} mutant embryos.

sufficient quantities to drive Dpp autoregulation and expand *dpp* transcription.

sdc and *trol* have both been implicated in BMP and/or Dpp signaling in other contexts (Fisher et al., 2006; Lindner et al., 2007), making them especially attractive candidates for an epidermal Dpp signaling antagonist. A sink for epidermal Dpp could also function as an antagonist of Dpp signaling in the mesoderm. To test this, we examined the specification of embryonic cardiac cells, labeled by the *B2-3-20* enhancer trap (Figure 3.7A) (Bier et al., 1989). *sdc* is required for proper cardiac cell specification, a phenotype which is enhanced in *dpp* mutant heterozygotes (Knox et al., 2011). In *sdc* mutants, there is a failure to specify cardiac cells in embryonic hemisegments (Figure 3.7B), and this loss is sensitive to Dpp levels (Knox et al., 2011). Loss of function of *raw*, an antagonist of JNK in the epidermis (Bates et al., 2008; Byars et al., 1999) leads to overspecification of cardiac cells during dorsal closure due to ectopic Dpp signaling (Figure 3.7C) (Yang and Su, 2011). *mmy* mutants, despite ectopic epidermal *dpp* expression, do not share this phenotype; in fact, cardiac cells of *mmy* loss-of-function embryos

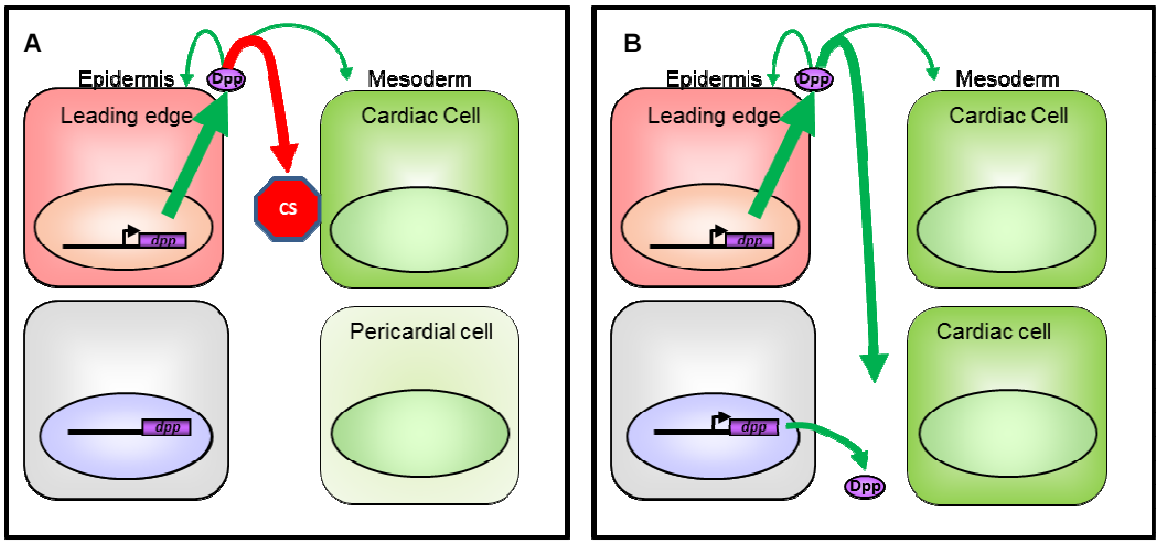

Figure 3.6. Model for CS-mediated embryonic Dpp antagonism. (A) Dpp secreted from the leading edge of the epidermis. It signals back to the leading edge cell to promote further *dpp* transcription and to the mesoderm to specify cardiac cell fate. Chondroitin sulfate, synthesized in the mesoderm, prevents excess Dpp signal from reaching the dorsolateral epidermis and mesoderm. (B) If the chondroitin sulfate sink is lost, Dpp access to dorsolateral epidermis and mesoderm are unrestricted; excess signaling results in ectopic *dpp* transcription and ectopic specification of cardiac cells.

exhibit a cardiac gap phenotype reminiscent of *sdc* mutants (Figure 3.7D). We suspect that this is the result of decreased HS and CS synthesis in *mmy* mutants leading to defective *sdc* glycosylation and signaling function, as *sdc* is a necessary cofactor of mesodermal *dpp* signaling (Knox et al., 2011). *wand* RNAi embryos, on the other hand, have cardiac cell gain (Figure 3.7E), reminiscent of *raw* embryos, which have too much Dpp signaling in the mesoderm (Klinedinst and Bodmer, 2003; Yang and Su, 2011). In this case, *wand* loss of function depletes the sink molecule CS, while leaving HS-modified Sdc intact to carry out necessary mesodermal Dpp signaling and cardiac specification.signaling and cardiac specification.

We tested the roles of *sdc* and *trol* both singly and together in antagonizing epidermal Dpp through analysis of mutant larval cuticles. The role of *sdc* in mesodermal Dpp signaling plus its expression in cardiac cells makes it an attractive candidate as the mesoderm-expressed Dpp sink. However, *sdc* mutant embryos do not have cuticle defects associated with ectopic *dpp* transcription, nor do they exhibit ectopic epidermal *dpp* transcript *in situ* (data not shown). Thus,

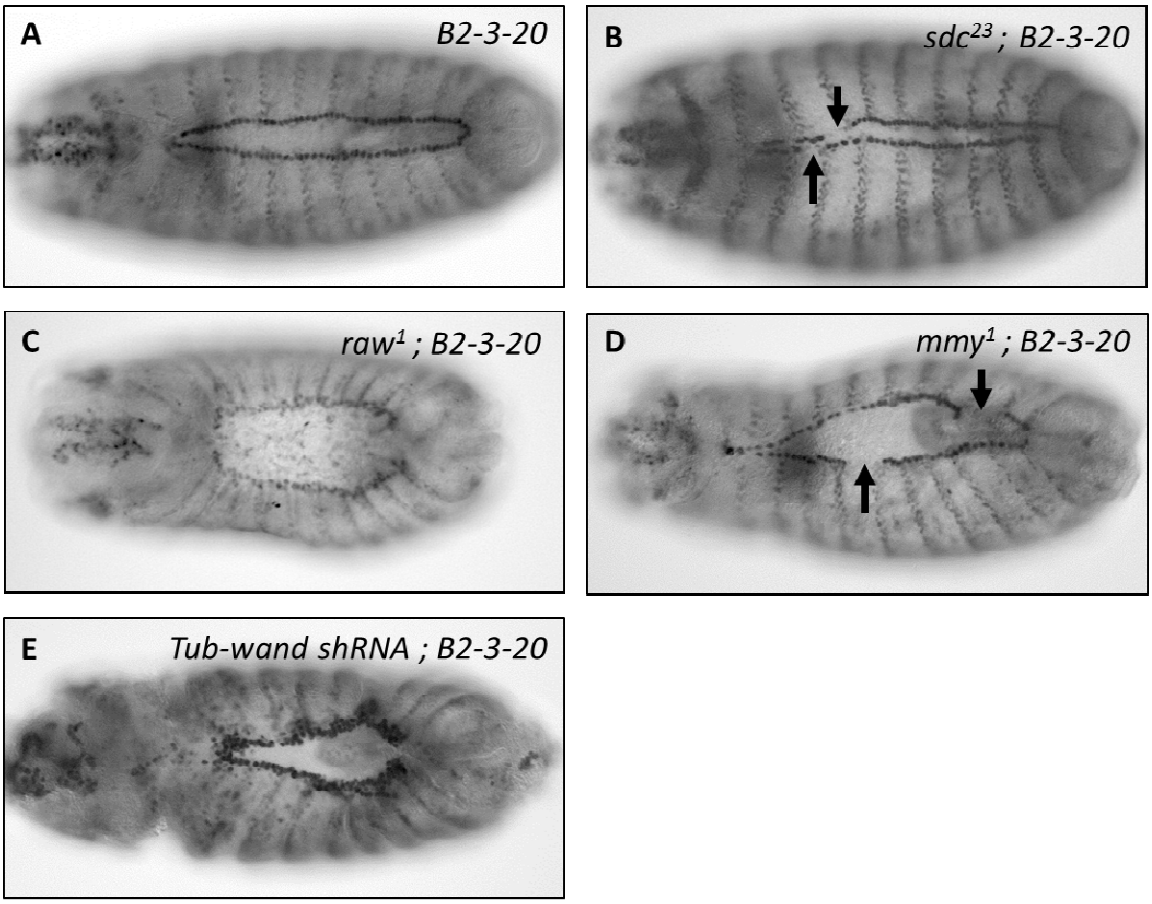

Figure 3.7. Cardiac cells, visualized with anti-β-galactosidase. (A) Cardiac cells in a wild-type embryo; these cells lie direcly underneath the LE epidermis. (B) Hemisegment loss of cardiac cells occurs in *sdc*^{23} mutants. Genetic interactions between *sdc* and *dpp* mutants suggest that *sdc*, possibly in concert with Dpp signaling, is required for cardiac specification. (C) *raw*^{1} mutants exhibit overspecification of cardiac cells due to ectopic Dpp signaling. (D) *mmy*^{1} mutants have hemisegment loss of cardiac cells, similar to *sdc* mutants. This may be a result of *mmy* mutants having defective *sdc* function. (E) *Tub-wand shRNA* embryos have overspecification of cardiac cells. The differences in *mmy*^{1} and *wand shRNA* phenotypes may be due to the fact that *mmy* mutants have disrupted *sdc* function due to loss of both CS and HS. Sdc in *Tub-wand shRNA* embryos would have decreased CS attachment, but would retain HS and be capable of participating in Dpp signaling.

while *sdc* is an important effector of mesodermal Dpp signaling, it is not the Dpp sink. Similarly, *trol* mutant embryos did not exhibit cuticle defects associated with ectopic Dpp signaling, nor did we observe ectopic expression of *dpp* in the epidermis (data not shown). Last, we tested if the signal antagonist may not be tied to any particular protein, but is due to a combination of CS-modified proteins; thus, the overlapping cardiac expression domains of *sdc* and *trol* could compensate for the loss of one or the other. To test this model, we examined cuticle phenotypes and dpp expression in *trol sdc* double mutant embryos. While nearly 50% of double mutant animals failed to deposit a cuticle, we did not observe *mmy*-like cuticles or *dpp* expansion in *trol* mutants, *sdc* mutants, or *trol sdc* double mutants in excess of background levels (data not shown). We did not study the effects of *kon-tiki* or *multiplexin* loss of function, so one of these CS proteoglycans might still have a role in Dpp signal antagonism. The exact CS-modified protein(s) that enable the sink has yet to be identified.

Discussion

In this study, we have shown evidence of the first genetically defined localized signaling sink that converts a long-range signaling molecule into a short range one and have identified *wand* and *Gale* as Dpp signaling antagonists. These data suggest that CS is an important molecule functioning in early Drosophila development. Drosophila are reported to have high chondroitin-4-sulfate expression in ovaries (Pinto et al., 2004) There is little *de novo* heparan sulfate synthesis in embryos until about 10–12 hours AEL; there is a significant component of CS present in the embryo at this point, though whether it is maternally deposited or made *de novo* is unknown (Pinto et al., 2004). CS-C is only detected in trace amounts in Drosophila larvae, and chondroitin during embryonic and adult life is 4-sulfated or unsulfated (Pinto et al., 2004). CS is more prevalent than heparan sulfate at all stages in Drosophila development, having nearly equal levels in embryos, and approximately 20:1 and 10:1 ratios of CS to HS in 3rd instar larvae and adults, respectively (Toyoda et al., 2000).

We have previously noted in wild-type embryos that Mad phosphorylation rapidly decays in the epidermis during dorsal closure in a *mmy*-dependent fashion (Humphreys et al., 2013).

Interestingly, the onset of expression of *wand* in the mesoderm adjacent to the leading edge coincides with this decay; further work will determine if CS synthesis might be the critical step in transitioning epidermal Dpp from a long-range to a short-range signal during dorsal closure. Experimental evidence has suggested that altering the heparan/chondroitin balance may be important in transitioning CS from a signaling mediator to a signaling sink. Mammalian Perlecan is involved in FGF delivery, and it can be modified by CS and HS. However, FGF is not released to its receptor if Perlecan is highly CS-modified; thus, CS may mark the change between a signaling effector and a sink in this protein (Smith et al., 2007). Unlike in the dorsolateral epidermis, the wing disc is an area of long-range Dpp signaling (Lecuit et al., 1996; Nellen et al., 1996; Schwank et al., 2011). Though *wand* expression would likely lead to CS synthesis in the wing disc, this does not transition to Dpp to a short-range signal in this context; thus the presence of CS does not strictly define short-range signaling. Context is very important in determining the interactions between CS proteoglycans and extracellular signaling molecules, and timing, dosage, sulfation, and other factors can determine whether the effect of a proteoglycan is positive, negative, or neutral on extracellular signals (Bai et al., 1999; Li et al., 2010; Mizumoto et al., 2013; Olivares et al., 2009; Shintani et al., 2006).

The embryonic epidermis is not the only tissue where Dpp signal is restricted to a short range. A similar restriction of Dpp signaling is seen in the stem cell niche of the testis, where Dpp expressed in somatic cells of the testes maintains the germline stem cells (Kawase et al., 2004; Michel et al., 2011; Song et al., 2004). Whether signaling range in this context is restricted by chondroitin sulfate is unknown.

It may at first be surprising that *Gale* and *wand* mutants were not generated in the Heidelberg screens for mutants affecting the embryonic pattern of cuticle (Jürgens et al., 1984). After all, this was the screen that generated alleles of *dpp* antagonists, including *mmy* and *raw* (Bates et al., 2008; Byars et al., 1999; Humphreys et al., 2013). This could be due to the fact that *wand* is maternally deposited, and this maternal transcript may be sufficient to complete embryonic patterning in the absence of zygotic-encoded *wand*. In fact, the two alleles of *wand* that we tested failed to reproduce the *mmy* mutant phenotype. The phenotypes of *Gale* and *mmy*

mutant embryos might be less severe than those seen in *wand* RNAi embryos for a few different reasons. First, there is a salvage pathway for hexosamines (including GalNAc) that allows them to reenter the pathway downstream of *mmy* and *Gale* (Vocadlo et al., 2003). As *wand* functions downstream of salvage, GalNAc recovered in the salvage pathway would still be blocked from being polymerized into CS in the RNAi embryos.

A second factor could be enzymatic redundancy. It is notable that *wand* is not the only predicted chondroitin synthase in the Drosophila genome. *CG9220* is predicted to encode an enzyme with similar function to *wand* (Wilson, 2002). Protein domain prediction identifies a chondroitin N-acetylgalactosaminyltransferase domain. Further study will determine if the expression patterns of *CG9220* and *wand* are complimentary or overlapping. Mice null for *wand* homologue *Css2* have no obvious phenotypic or morphological changes, perhaps due to redundancy of CS synthesis enzymes (Ogawa et al., 2012). Indeed, loss-of-function of *Css2* decreases the quantity of CS chains exceeding 10 kDa in weight, but does not affect the total number of CS chains attached to core proteins (Ogawa et al., 2012). Similarly, mutations in *Gale* may have partially penetrant effects due to potentially redundant enzymatic activities from the product of *CG5955*, a predicted UDP-glucose 4-epimerase (Flybase). However, *CG5955* is not found in embryos until 16 hours after egg lay, according to the modENCODE RNA expression database (Celniker et al., 2009).

Tangential to the goal of this study was the observation that Dlp levels are decreased when glycosylation is inhibited in *mmy* mutant embryos. In many cases, glycosylation has been demonstrated to be dispensable for the interaction of a proteoglycan and signaling ligands. The *C. elegans* glypican LON-2 is an antagonist of BMP signaling (Gumienny et al., 2007; Taneja-Bageshwar and Gumienny, 2012). LON-2 has two functional domains that are able to bind to BMP; the C-terminal domain contains heparan sulfate attachment sites, while the N-terminal domain has no attachments but is able to bind to BMP and inhibit signaling independent of the C-terminal. In Drosophila the protein Dally does not require HS attachment to complete its role in Dpp signaling, though HS does make it more efficient (Kirkpatrick et al., 2006b). Expression of a *dally* construct with all HS attachment sites removed was able to partially rescue some signaling

defects in *dally* mutant animals, especially in wing formation (Kirkpatrick et al., 2006b). These data are complicated by the fact that the authors were unable to determine how much Dally was generated by the rescue construct. As partial loss of HS chains may decrease glypican stability, future studies on the role of HS and CS modification on protein function and interaction should take care to ensure that alterations in HS and CS levels do not result in unintended reduction in protein stability. Overall, the data presented here indicate that CS production may be utilized to create an extremely sharp signaling gradient, and this mechanism may be used in other organisms and developmental systems toward the same end. Increasing CS production might even be explored as a potential therapy for diseases caused by an overabundance of BMP signaling activity.

CHAPTER 4

FUTURE EXPERIMENTAL PROPOSALS FOR THE STUDY OF CHONDROITIN SULFATE IN DEVELOPMENTAL SIGNALING

While much has been published concerning the role of glycosaminoglycans (GAGs) in regulation of diverse signaling molecules, including BMPs, these publications have focused primarily upon HS (Baeg et al., 2001, 2004; Desbordes and Sanson, 2003; Fujise et al., 2003; Han et al., 2004; Rapraeger et al., 1985; Saunders et al., 1997). Moreover, information concerning the role of CS proteoglycans during development has been very limited. CS proteoglycans have potential effects on cytokinesis and polarity as well as neural stem cell renewal (Mizuguchi et al., 2003; Sirko et al., 2007), and the work in the previous chapters outlines a requirement for CS to antagonize Drosophila Dpp signaling. CS was not previously known to have any direct function in Dpp signaling, and regulation of Dpp signaling through GAGs was thought to be mainly carried out by heparan sulfate proteoglycans; Dally in particular is known to shape Dpp signaling gradients in wing discs (Akiyama et al., 2008; Desbordes and Sanson, 2003; Fujise et al., 2003; Jackson et al., 1997). That many classical HS-binding factors have recently been found to interact with CS (Deepa et al., 2002; Lyon et al., 2002; Mizumoto et al., 2013) underscores the necessity for more information about the nature and effects of these interactions. Clearly, CS plays a greater role in BMP signaling, and may have applications in treating human diseases with abnormal BMP signaling, including fibrodysplasia ossificans progressive (FOP) and cancer.

FOP is the best characterized human disease caused by excess BMP signaling. This highly debilitating disease causes the victims' soft tissues to be slowly ossified to bone in response to injury. FOP is rare, with its incidence estimated at approximately 1:2,000,000

(Kaplan et al., 2008). Though the disease-causing allele can be inherited in an autosomal dominant fashion, the majority of FOP cases arise due to *de novo* mutation. An R206H mutation in the Activin receptor type-1 (ACVR-1) is the cause of most cases (Dinther et al., 2010; Shore et al., 2006), though the disease can also result from a frame-shift mutation in the receptor gene (Dinther et al., 2010; Fukuda et al., 2008; Kaplan et al., 2008). In the progression of FOP, ACVR-1 mutation results in hyperactive BMP receptor activity and a breakdown of BMP negative feedback, which results in overproduction of BMP-4 mRNA and protein (Dinther et al., 2010; Kaplan et al., 2008). The use of exogenous heparan or the alteration of endogenous HSPG has been suggested as a therapy to quench BMP signaling and reduce the severity of FOP (Jiao et al., 2007; O'Connell et al., 2007). We similarly propose that CS induction may help quench BMP-4 signaling and lessen tissue ossification postinjury in FOP patients.

Many cancers have an upregulation of BMP signaling, whether through gain in BMP gene copies (Alarmo et al., 2006) or transcriptional regulation (Rothhammer et al., 2005). In short, BMP signals are increased in several cancers, though the effects are BMPs in these cancers can either advance or inhibit cancer progression; activity of Dpp homologue BMP-2 especially contributes to tumor progression (Singh and Morris, 2010). The processes of angiogenesis and metastasis, to which BMPs contribute, are very complex, and the effects of BMPs may be either primary or secondary (Singh and Morris, 2010). We speculate that, as CS plays an important role in Dpp signaling regulation in the Drosophila embryonic epidermis, BMP signaling during complex processes such as cancer angiogenesis and metastasis may be similarly influenced by CS. Though most studies investigating GAG therapy as a treatment for cancer have focused on HS and hyaluronan (Yip et al., 2006), there may be unrecognized therapeutic value of chondroitin as a molecule to prevent metastasis, growth, or angiogenesis of certain tumor types. Prior to answering the questions of potential CS therapeutics in human BMP disease, we must fully understand the interactions between CS and BMP signaling.

There are four main questions that remain regarding the interaction of chondroitin sulfate (CS) with Decapentaplegic (Dpp) signaling antagonism in the Drosophila dorsolateral epidermis. 1) Does the transferase encoded by *wanderlust (wand)* function to synthesize CS? 2) Is there a

direct physical interaction between CS and Dpp? 3) Does CS function as a Dpp signaling antagonist in other contexts during Drosophila development or homeostasis and even in other organisms? 4) What are the specific CS-modified proteins functioning to antagonize Dpp signaling in the Drosophila embryonic epidermis? In this chapter, I will explore potential experimental methods to address these questions and the advantages and drawbacks of the various protocols.

Procedures for the physical quantification of CS in Drosophila embryos

Methods for quantification of CS

Though BLAST analysis clearly suggests that *wanderlust* (*wand*) encodes a chondroitin sulfate synthase, I was unable to clearly demonstrate that chondroitin sulfate levels are reduced when *wand* function is depleted in the early Drosophila embryo. Three different methods were utilized in an attempt to quantify Drosophila CS synthesis. Ultimately, all were unsuccessful. The first attempt was by western blotting utilizing the CS-56 antibody; whether it is able to detect chondroitin-4-sulfate (CS-A), which is the sole form of CS that is expressed in *D. melanogaster,* is controversial (Avnur and Geiger, 1984; Pinto et al., 2004; Yi et al., 2012; C. Mencio, personal communication, March 2014). In western blot analysis, I noted that the antibody successfully detected purified CS-C, but was unable to detect CS-A (data not shown). This suggests that the CS-56 antibody is unsuitable for use in *D. melanogaster,* as Drosophila do not synthesize CS-C. Anti-chondroitin 4S (clone 2H6) can efficiently detect CS-A (chondroitin-4-sulfate) (Yi et al., 2012), which is the only type of CS known to be expressed in Drosophila (Sugiura et al., 2012). Anti-chondroitin 4S antibodies are available solely from Amsbio and may prove useful in quantification of CS synthesis in western blot analysis, as well as have potential applications visualizing CS localization in whole-mount embryos *in situ*.

The second method whereby I attempted to quantify CS synthesis in Drosophila was by DEAE column purification of GAGs followed by chondroitnase digestion and mass spectroscopy. To test if genetic manipulation might have an effect upon CS synthesis, 200 embryos were ground and incubated for 60 minutes in NP-40 lysis buffer and spun down 10 minutes at

maxiumum speed. The supernatant was passed three times through a 250 ul DEAE sepharose column, which binds to negatively charged species such and heparan, chondroitin, and nucleic acids. Columns were washed five times, and eluted in 1.5 M NaCl. The eluate was desalted in an Amicon Ultra tube and then incubated at 37° over night after adding chondroitinase ABC, an enzyme that breaks chondroitin chains down into disaccharide components (Prabhakar et al., 2005). The samples were then boiled for 1 minute and then spun down for 10 minutes to pellet the protein. The supernatant was then collected and subjected to mass spectroscopy analysis. However, no chondroitin sulfate disaccharide parental peaks were observed in the spectra.

On the third quantification attempt, I utilized a modified protocol employed by Pinto et al. in quantifying GAGs in *Drosophila* embryos (Pinto et al., 2004). 500 embryos were submerged in 3 ml of acetone and incubated for 12 hours at 4℃. The acetone was dried off at 60℃, and then embryos were suspended in papain buffer (50 mg papain, 5 mM EDTA, and 5 mM cysteine in 10 ml of water) and incubated for 12 hours at 60℃. The embryos were pelleted in a centrifuge (2000 x *g*, 10 minutes, 25℃), and the supernatant was reserved. 10 ml of fresh papain buffer was added to the pellet, and the same procedure was repeated until 3x10 ml aliquots of supernatant were collected. Supernatants for each sample were pooled, and the GAGs were precipitated by addition of 90 ml of ice-cold 95% ethanol. Samples were incubated for 12 hours at 4℃. Precipitate was collected by centrifugation (2000 x *g*, 10 minutes, 25℃) whereupon the supernatant was removed and the precipitate allowed to air-dry. Samples were resuspended in 1 ml water and digested overnight with chondroitinase ABC and subsequently analyzed by mass spectroscopy. No chondroitin sulfate-associated parental peaks were detected in the resulting spectra. It is possible that this protocol could be more sensitive if parent flies are fed a diet containing $Na_2{}^{35}SO4$, which would cause the embryonic HS and CS to be labeled with ^{35}S. $Na_2{}^{35}SO4$ feeding was employed in the original study.

Analysis of Wand function

Ultimately, loss of function of *wand* may not lead to a detectable depletion of chondroitin sulfate due to potential redundancy of chondroitin sulfate synthesis between the enzymes

encoded by *wand* and *CG9220*. In this case, the phenotype of *wand* loss of function may be due to the specific cardiac-restricted expression of *wand* during Drosophila dorsal closure. *wand* may have a specific function in this tissue at this time point above and beyond the function performed by general embryonic chondroitin sulfate, which is maternally deposited in high levels into oocytes (Pinto et al., 2004).

Because of the difficulty in assaying the loss of CS directly in *wand* RNAi embryos, focus should be shifted to the biochemical function of the enzyme encoded by *wand*. The activity of Wand protein can be determined just as the human ChSy1, ChSy2, and ChSy3 were assayed (Kitagawa et al., 2001; Yada et al., 2003a, 2003b). The human homologue of *wand*, *ChSy2*, is predicted to have a transmembrane domain that places the active site inside the Golgi apparatus lumen (Yada et al., 2003a), though at least one splice variant is localized to the mitochondria (Kuroda et al., 2012). The enzyme must be solubilized in order to perform enzymatic activity tests.

To overcome the issues associated with transmembrane localization, a truncated version of gene sequence that encodes the active site can be ligated downstream of an insulin targeting sequence and Protein A-encoding sequence. This construct can then be inserted into a plasmid and introduced into COS cells. COS cells expressing the chimeric protein will secrete it into the culture medium, and the protein can be purified by passing through a column containing anti-protein A-coated beads. Purified chimeric enzyme can then be assayed for enzymatic functions in CS synthesis, as described elsewhere (Kitagawa et al., 2001; Yada et al., 2003a, 2003b). Wand should be tested for both β1,3-GlcUA transferase and β1,4-GalNAc transferase activities, as these are the activities present on homologue Css2 (Yada et al., 2003a). As a negative control for this experiment, COS cells would be transformed with a plasmid encoding the insulin targeting sequence ligated to the Protein A-encoding sequence; culture supernatant from these cells should not have increased β1,3-GlcUA transferase and β1,4-GalNAc transferase activities. A negative control for this experiment would be a plasmid that contained only the insulin targeting sequence and Protein A-encoding sequence. For a positive control, COS cells could be transformed with a plasmid encoding a truncated version of human Css2, which has been ligated

to an insulin targeting sequence and a Protein A-encoding sequence, as previously described (Yada et al., 2003a).

Proposed methods to test for a physical interaction between CS and Dpp

In the previous chapter, it was demonstrated that loss of function of the enzymatic machinery required for CS synthesis results in ectopic *dpp* transcription and expanded Dpp signaling. The exact cause of the *dpp*/Dpp expansion is unknown at this time, but we have proposed that CS acts as an extracellular sink that binds Dpp and interferes with its signaling through its receptors. In order to test this model, an approach to directly test for a physical interaction between Dpp and CS is needed.

Procedures for the visualization of Dpp *in situ*

Due to the difficulty of raising antibodies to detect Dpp *in situ* (Lecuit and Cohen, 1998), live visualization of Dpp might be accomplished through a Dpp-GFP fusion protein. Dpp-GFP under the transcriptional control of the UAS promoter has been successfully utilized for studies in Drosophila to investigate the shape of Dpp gradients in wing discs and how glypicans can alter those gradients (Teleman and Cohen, 2000). However, the existing line is somewhat problematic, in that signaling gradients are sensitive, and changes in levels of receptors or ligands can both have effects on the shape of the gradient. In systems where Dpp-GFP is induced by Gal4 expression, the Dpp-GFP expressed would be in addition to the endogenous locus, and might be sufficient to disrupt the interactions we are trying to observe. These issues might be circumvented by generation of a Dpp-GFP fusion protein that replaces the endogenous *dpp* locus. Furthermore, eliminating the need for Gal4 to drive UAS:Dpp-GFP expression simplifies genetics for further manipulations, or would allow use of the UAS-GAL4 system to drive other transgenes independent from Dpp-GFP expression.

Procedures for the visualization of chondroitin *in situ*

Live visualization of chondroitin through GFP-antibody fusion expression

One novel method used to visualize GAGs *in vivo* was developed in *C. elegans*. The worms expressed in their coelomocytes, cells of the innate immune system, a GFP-fused antibody specific to particular HS structures (Attreed et al., 2012). The antibody is secreted from the coelomocytes and then accumulates on cells and tissues that have the corresponding HS epitope, allowing its localization to be visualized. Such a system might work in Drosophila to detect CS, but potential unanswered questions include where to drive expression of CS (though hemocytes might make a good candidate) and what antibodies to use for CS detection. The lack of good antibodies to detect CS *in vitro* or *in vivo* in Drosophila requires special attention, but if new antibodies are found to work effectively, this could be an effective method to visualize CS localization in Drosophila. The best candidate might be anti-chondroitin-4-sulfate, encoded by plasmid 2H6, but this antibody should be tested *in vivo* for efficient detection of CS, as described earlier in this chapter. Transgenic antibody expression in living animals might not be effective for colocalization experiments, as antibody binding to CS is likely to disrupt the ability of CS to interact with other extracellular molecules by steric hindrance or competing for binding sites. Nevertheless, it may prove useful as a method to analyze the dynamic expression of chondroitin sulfate proteoglycans (CSPGs) during Drosophila development.

Vizualization of chondroitin by Staudinger-Bertozzi ligation

Staudinger-Bertozzi ligation is an additional method whereby CS can be visualized in live embryos. A sugar with a specific azide moiety is introduced into the embryo, where it can be incorporated into glycans via the hexosamine salvage pathway. A triarylphosphine can then be conjugated to the azide moiety in a mild reaction that takes place in aqueous solutions (Saxon and Bertozzi, 2000). The azide is incorporated into embryonic CS; it can be ligated to a specific FRET-based phosphine with an attached fluorophore and compatible FRET quencher. Following Staudinger ligation, the quencher is released into solution allowing the fluorophore to be turned on (Hangauer and Bertozzi, 2008).

N-azidoacetylgalactosamine, or GalNAz, is the azide moiety that can be utilized to create UDP-GalNAz through the GalNAc scavenging pathways (Hang et al., 2003). In order to enable efficient labeling of CS with GalNAz, sufficient substrate must be introduced into the Drosophila embryonic cells. UDP-GlcNAc and UDP-GalNAc (and their azide counterparts) cannot be absorbed across the cell membrane due to a negative charge and lack of membrane transporters; UDP-GlcNAc is synthesized in the cytoplasm from glucose and is then transported into the ER and Golgi through specific transporters which are localized there and then utilized there to carry out glycosylation (Perez and Hirschberg, 1985). UDP-GalNAc can then be incorporated into GalNAc-containing glycosylation modifications; GalNAz, if present, can substitute for GalNAc in these glycosylmodifications. There are two methods that might be used to introduce GalNAz into embryos. The first option is feeding GalNAz directly to females, who would then incorporate it into oocytes. If the amount of GalNAz delivered into oocytes in insufficient for labeling, then an alternate second option would be to inject GalNAz as a bolus into early syncytial blastoderms. In either case, GalNAz can be utilized to make UDP-GalNAz through the GalNAc salvage pathway.

It is important to note that GalNAz feeding or injection will not solely label CS; though GalNAz will be incorporated into CS, it will also be incorporated into mucin-type O-linked glycoproteins (Hang et al., 2003). This may cause difficulty in distinguishing mucin-type O-linked glycoproteins from chondroitin sulfate proteoglycans. However, mucin-type O-linked glycoproteins only can incorporate GalNAz in a covalent linkage to hydroxyl group of serine or threonine residues (and thus may contain a maximum of one GalNAz per oligosaccharide), whereas a CS could contains up to 50% GalNAz, if no other endogenous sources of GalNAc are present and GalNAz is used exclusively (Hang and Bertozzi, 2001). Thus, large differences between the signal intensities should make CS easier to detect.

Combination of chondroitin and Dpp visualization to determine colocalization

Physical proximity between chondroitin and Dpp could then be assayed by injecting GalNAz into early syncytial blastoderm embryos which have their endogenous Dpp replaced by a Dpp-GFP protein as proposed earlier in this chapter. GFP and the phosphine-attached fluorophore must have distinct wavelengths of emission so as to avoid spectral bleed-through.

One last method might provide *in vitro* evidence of an interaction between Dpp and CS. Previously, binding affinity between Bmp-4 and 4,6-disulfated chondroitin has been assayed through fluorescence correlation spectroscopy (Miyazaki et al., 2008). This microscopy method can track movement and diffusion, due to Brownian motion, of fluorescently labeled molecules and thus calculate a diffusion coefficient for the molecules. As proof of principle, in a published study the diffusion of fluorescine-labeled 4,6-disulfated chondroitin is slowed when Bmp-4 is added to the solution; this indicates a physical interaction between the two molecules (Miyazaki et al., 2008). Fluoresceinamine-labeled sodium chondroitin sulfate A can be obtained from Cosmobio Inc. (catalog#CSR-FACS-A1). Recombinant Dpp can be synthesized and properly processed posttranslationally in Chinese hamster ovary cells without likely complications (Israel et al., 1992). In addition to testing for interaction with CS-A, the interaction of Dpp with CS-O (unsulfated CS) should also be tested, and research indicates that chondroitin in Drosophila is often undersulfated (Pinto et al., 2004).

Proposed chemical, enzymatic, and genetic methods to alter CS synthesis

Many things could be learned about the role in CS in shaping signaling gradients if there were a method to boost and antagonize CS synthesis. This could provide new insight into mechanisms of gradient formation, tissue-specific factors that contribute to gradient shape, and other signaling factors which are affected by CS. CS may play an unappreciated role in Dpp signal regulation in other tissues. This section will discuss methods to alter CS synthesis *in vivo*, the potential applications of these methods, and the potential pitfalls and complications of altering CS synthesis.

Utilizing xylosides to promote and antagonize CS synthesis

One possibility to investigate the effects of chondroitin sulfate on early Dpp signaling is through the use of xylosides. Heparan and chondroitin formation is initiated by transferring glucose or galactose from their corresponding UDP-linked counterparts. Xylosides are glycosides that are generated from xylose and can serve as acceptors of glucose or galactose transfer. In 1973, it was noted that *p*-nitrophenyl β-D-xyloside was able to serve as substrate for galactosyl transferase I, leading to the initiation of synthesis of free glycosylaminoglycan chains (Okayama et al., 1973). Since this time, xylosides with varying structures have been tested for their ability to promote or antagonize chondroitin, heparan, and dermatan synthesis (Cambiazo and Inestrosa, 1990; Carrino and Caplan, 1994; Fritz et al., 1994; Gibson et al., 1977; Klein et al., 1989; Lugemwa et al., 1996; Tsuzuki et al., 2010; Vassal-Stermann et al., 2012). It is also worth noting that any chondroitin or heparan synthesis that is initiated by xylosides will not be bound to a core protein, but will be free-floating in the extracellular matrix (or the culture medium, depending on the protocol) (Okayama et al., 1973; Sobue et al., 1987). In choosing a xyloside, factors to consider are toxicity, rate of promotion or blocking of CS synthesis, and bleed-over of effects on other GAG moieties. For instance, consider 4-deoxy-4-fluoroxylosides. 4-deoxy-4-fluoroxylosides have relatively low toxicity, having no negative effects on cells at 300 uM and lower concentration (Tsuzuki et al., 2010). They are effective at blocking GAG synthesis, as they lack an acceptor hydroxyl group at C-4 in order to allow enzymatic initiation and extension of GAG chains (Tsuzuki et al., 2010). Thus they serve as competitive inhibitors to extension of xylose bound to proteins, and many core proteins will remain naked under 4-deoxy-4-fluoroxyloside treatment conditions. These xylosides are effective at blocking both heparan and chondroitin synthesis and are ideal in cases where the consequences of total GAG loss are of interest. Considering these four points of xyloside function assists in selecting the proper tool for a xyloside assay. One common pitfall in selecting a xyloside for treatment is to select one with low tissue permeability, as some early studies that explored the capabilities of xylosides to accept galactose from UDP-galactose were performed in cell-free systems (Robinson and Robinson, 1981; Sobue et al., 1987)

Also of note, while many xylosides preferentially prime synthesis of CS chains, β-D-xylosides that have two fused aromatic rings can efficiently prime HS chain synthesis (Fritz et al., 1994). Though β-D-xylopyranosides (or O-glycosides) are typically used in order to promote CS synthesis, C-xylopyranoside incubation for 14 days increased GAGs found in in the extracellular matrix in human fibroblast culture by 15 fold. C-xylopyranoside may be useful for some applications, as it has a higher metabolic stability than traditionally used β-xylosides (Vassal-Stermann et al., 2012). In cell culture situations, β-D-xyloside causes the initiation of several CS chains that have normal structure and sulfation patterns, in comparison to their CSPG counterparts, but differ in that the xyloside-linked CS chains are shorter (Carrino and Caplan, 1994).

Despite the strength of xylosides in specifically targeting GAGs, there are some not insignificant drawbacks that may complicate their effective use in studying the effects of CS on Dpp signaling. Due to the rapid changes that take place during development, and the fact that xyloside treatment would not eliminate CS synthesis but would only eliminate CSPG synthesis, it may not be fruitful to pursue xyloside injection in Drosophila embryos. If chondroitin sulfate is truly acting as a sink for Dpp, then it might likely have that same effect whether found in the ECM or bound to a CSPG such as Sdc or Trol. Xylosides may still have some value to study Dpp antagonism at later time points, once the free-floating CS has time to clear from the system. More data are required to estimate the time required for this clearance to occur in Drosophila.

That being said, one piece of data about the interaction of Decorin with TGF-beta signaling suggests that xyloside treatment may still be worth study. Chinese hamster ovary cells are known to produce chondroitin sulfate. In one experiment, the cells were made to overexpress Decorin, which is a chondroitin/dermatan sulfate proteoglycan. Decorin expression led to a decrease in cell growth due to a decrease in TGF-beta signaling. In fact, it was found that Decorin had a strong affinity for TGF-beta. Interestingly, TGF-beta stimulates expression of *decorin*, suggesting that the CSPG Decorin might be part of a negative feedback pathway for TGF-β signaling (Yamaguchi et al., 1990). The core protein of Decorin itself was found to be inhibitory, so the attachment of CS and DS was not necessary for inhibition. In Chapter 3, data

were presented that suggested that truncation of HS might result in decreased stability of the glypican Dally-like. If this is the case, then xyloside treatment, which causes decreased HS and CS synthesis on core proteins, could result in lower stability of those core proteins. Similar mechanisms could also exist for chondroitin sulfate, though I could not find any specific examples in the literature.

Utilizing modified xylose to block GAG synthesis

Xyloside treatment is not the only method whereby embryonic CS can be eliminated. As HS and CS chains are synthesized on xylose, chain synthesis can be blocked by introducing a xylose that is incompatible with chain initiation; by this principle, 4-azido-4-deoxyxylose is an effective blocker of GAG synthesis. A chain-terminating 4-azido-4-deoxyxylose can be introduced onto core proteins by introducing UDP-4-azido-4-deoxyxylose into the metabolic system (Beahm et al., 2014). Zebrafish embryos injected with 50 pmol of UDP-4-azido-4-deoxyxylose during the 1–4 stage had a reduction of HS and CS levels by 47□% and 77□%, respectively. Because synthesis of GAG is reduced, there are fewer concerns about issues with synthesized GAG chains being cleared from the system, as is the case for xyloside treatment. Though the treatment described here is most effective at reducing CS synthesis, a significant HS reduction remains, so there is the potential for off-target or confounding effects.

Utilizing chondroitinase ABC to enzymatically destroy CS *in vivo*

In order to determine the effects of loss of CS, enzymatic methods of CS digestion may be of use. The most straight-forward approach would involve soaking embryos in a chondroitinase ABC solution. However, though many permeabilization protocols have been demonstrated to allow the uptake of small molecules up to 1 kDa in size to be taken up in the embryo (Rand et al., 2010), far less is known about the ability of permeabilized embryos to take up a molecule of molecular weight 120 kDa such as Chondroitinase ABC. A method with more potential applications and that would allow temporally and spatially restricted analysis of CS function would be generation and use of a UAS-chondroitinase ABC transgenic line. The line

could be generated by insertion of a modified chondroitinase-encoding plasmid into attP sites using a φC31-based integration system (Bischof et al., 2007; Prabhakar et al., 2005). Studies have shown that the Chondroitinase ABC I from *Proteus vulgaris* can be secreted from mammalian cell culture when certain N-glycosylation sites are eliminated by site-directed mutagenesis (Muir et al., 2010). This mutagenesis may be sufficient in order to allow secretion from Drosophila cells as well, but this will need to be tested by transfection in S2 cells.

Increasing CS synthesis through induced expression of pathway components

Chondroitin sulfate serves as a sink in the context of the epidermis, and it may be able to function as a sink in other Drosophila tissues. An ectopic sink for Dpp in the wing disc might be created by generating more CS, which could be detected by a reduction of P-Mad localization in the wing disc. An area that exhibits long-range Dpp signaling might be altered to short-range Dpp signaling when additional CS is introduced into the area. One way to accomplish this would be through overexpression of *wand* mRNA, leading to a higher concentration of enzyme to catalyze synthesis of chondroitin sulfate. 3rd instar wing discs would work well for this experiment, as Dpp functions as a long-range signaling in this tissue, and there is no known function for CS in the wing disc (Lecuit et al., 1996). However, all of the necessary components, and perhaps some other ones, must be present in order for CS synthesis to occur. For instance, an increase in CS synthesis in wing discs could not occur due to an increase in *wand* expression if *Gale* expression is insufficient to provide the additional UDP-GalNAc to meet demand. A functional increase in CS would require the presence of a CSPG in order to be the acceptor of the extending CS chain. Additional CSPG core protein expression would also be required if CS is already saturated in the wing disc.

When I attempted this experiment, transcription of *UAS:wand* under control of *vestigial:Gal4* could not be detected. The UAS site was located on the 3' terminal region of a *piggyback* transposon, designed to allow expression activation of adjacent genes, inserted upstream of *wand* (Thibault et al., 2004). A functional *UAS:wand* transgenic line is required in

order to proceed with this experiment.

Applications of chondroitin depletion

Testing if *wand* RNAi phenotype is due to physical interaction with other proteins

It is possible that the effects seen by *wand* depletion are not all due to loss of CS synthesis. (Though, in the case of *wand* it is likely that many of the phenotypes are, due to the fact that they are shared with *mmy* and *Gale* mutants.) Several enzymes that are involved in GAG synthesis have interactions with other proteins; thus, eliminating a particular enzyme may have unintended consequences in other systems that are unrelated to the downstream substrate in question. This has been documented for EXT1 and EXT2, which have function to synthesize HS (Senay et al., 2000). EXT2 catalyzes little to no HS synthesis in the absence of EXT1, but both have a synergistic effect when in complex to increase their synthesis beyond the sum of their activities (McCormick et al., 2000). Additionally, when the cytoplasmic tail of Uronosyl 5-epimerase was altered to change its localization from the Golgi apparatus to endoplasmic reticulum, 2-O-sulfotransferase was likewise relocalized (Pinhal et al., 2001). It is as of yet unknown what proteins interact with Wand, but *wand* mRNA depletion could result in effects in other pathways if this prevents proteins that associate with Wand on the Golgi membrane to fail to assemble. Utilizing either xylosides, a xylose analog, or CS-ABC to eliminate CS synthesis could answer the question whether the effects of *wand* loss of function on Dpp signaling are due to CS itself, or due to a possible confounding effect due to Wand being part of a protein complex.

Creating a *de novo* CS sink, or destroying an *in vivo* sink

Dpp signaling range varies dependent upon tissue and developmental time point in different Drosophila tissues. In the embryonic dorsolateral epidermis, as I have described here, Dpp signals at a short range. Long range signaling has been extensively studied in larval imaginal discs, where different concentrations of Dpp result in differential gene expression (Nellen et al., 1996). The other place where Dpp is restricted to short range is the stem cell hub of the

testes and ovary (Kawase et al., 2004; Shivdasani and Ingham, 2003; Song et al., 2004); Mad is phosphorylated in germline stem cells and gonialblasts, but remains unphosphorylated in all other cells of the testis (Kawase et al., 2004).

Dpp is made in the somatic hub cells, and P-Mad is observed in the adjacent germline stem cells. In order to test if the short-range localized Dpp signaling is a result of chondroitin sulfate acting as a Dpp sink, RNAi against *wand* and/or *CG9220* can be performed in the testes, followed by analysis for expansion of P-Mad regions. *wand* and *CG9220* are mostly absent from testes of 4 day old mated males (Celniker et al., 2009). However, it is of note that P-Mad detection in adult testes is strongest during the first 48 hours after adult flies eclose (Chang et al., 2013), so any major requirement for CS in the testes would most likely be seen earlier than day 4 after eclosion. *mmy, Gale,* and *sdc* have moderate expression in testes, while expression of *trol* remains low (Celniker et al., 2009). Because of the potentially redundant functions of *wand* and *CG9220* in CS synthesis, ideally both would be depleted with RNAi. Alternately, tissue-specific expression of chondroitinase ABC, as described earlier in this chapter, could be utilized. Chondroitinase ABC should be used with caution, as its diffusion in the extracellular space could suggest cell nonautomomous effects on systems that are autonomous. The CS sink could be present in one of several potential cell types (functioning as a cell autonomous or a cell nonautonomous sink). To this end, the UAS-Gal4 system could be used to induce tissue-specific depletion of chondroitin sulfate to determine which cell type(s) are important in Dpp signal antagonism in the testes. Depletion could be accomplished by wand shRNA expression or by expression of chondroitinase ABC. Depletion in germline stem cells could be accomplished under the control of *nanos-Gal4-VP16* (Van Doren et al., 1998). Depletion in hub cells can be accomplished by *Upd-Gal4* expression of shRNA (Zeidler et al., 1999) and cyst stem cells and cyst cells by using *ptc-Gal4* (Tazuke et al., 2002).

CSPGs of particular interest in Drosophila

There are four CSPGs that are known to be expressed in the embryonic Drosophila heart and may be of particular interest in determining the CS-modified factors that can modify Dpp

signaling range.

Multiplexin

Multiplexin (Mp) is a homologue of human collagens XV and XVIII, which are heparan/chondroitin sulfate proteoglycans; failure of the Drosophila protein to shift in gel after incubation with heparitinase suggests that it is CS modified only (Momota et al., 2011). Mp localizes primarily to the basement membrane of cells and is prominently expressed in many tissues, including the cardioblasts of the heart during embryonic stage 16. *mp* hypomorphs have phenotypes associated with Wg signaling failure, including wing defects, absence and/or abnormal fusion of tergites in the adult abdomen, and abnormal deposition of Wg protein itself in the provetriculus (Momota et al., 2011). Multiplexin is expressed in the cardiac cells near the time of dorsal closure completion and is thus not likely to play a large role in Dpp regulation during dorsal closure.

Terribly reduced optic lobes

terribly reduced optic lobes (*trol*) was named due to the reduction in imaginal disc size and abnormal brain morphology (Datta and Kankel, 1992). trol mRNA is expressed strongly in stage 15 embryonic cardiac cells (Friedrich et al., 2000). Trol is found in the extracellular matrix around the Drosophila lymph gland (Grigorian et al., 2013). Human Perlecan core protein binds to FGF7 ligand, and HS modification is dispensable for this modification (Mongiat et al., 2000).

Syndecan

Syndecan comes from the Greek “syndein,” which means “to bind together”; syndecan binds to growth factors and extracellular matrix extracellulary and intracellularly to actin (Bernfield et al., 1992). All syndecans are heparan sulfate proteoglycans, but many syndecans, including those found in Drosophila, also have attached chondroitin sulfate (Chanana et al., 2009; Gould et al., 1992; Rapraeger et al., 1985). Ligands that are bound to syndecan can be released into the ECM by cleavage of the syndecan ectodomain, potentially titrating the ligands away from their

cell surface receptors (Bernfield and Hooper, 1991; Kato et al., 1998).

Syndecans have been well characterized for their roles in migration of axons and cells in *D. melanogaster* and *C. elegans* (Johnson et al., 2004; Minniti et al., 2004; Rhiner et al., 2005; Smart et al., 2011; Steigemann et al., 2004). Axonal migration defects are primarily due to the role of *syndecan* in Slit/Robo signaling (Chanana et al., 2009; Johnson et al., 2004; Steigemann et al., 2004). There have been few studies documenting the role of *syndecan* on early development.

Kon-Tiki

The CSPG *kon-tiki*, the Drosophila homologue of CSPG4, is expressed in the dorsal vessel during dorsal closure in a shared pattern with *wand* (Hammonds et al., 2013; Tomancak et al., 2002, 2007). Though it has been studied for its role in myofiber targeting and muscle specification, potential roles in heart development and signal regulation have not been addressed (Pérez-Moreno et al., 2014; Schnorrer et al., 2007; Wolfstetter and Holz, 2012).

These four CSPGs, and any others which are discovered to play a role in the Drosophila heart, should be the focus of ongoing studies to determine the particular CSPG(s) that antagonize Dpp during Drosophila embryonic dorsal closure.

CHAPTER 5

ADDITIONAL EXPERIMENTS OF NOTE

Tests for transcriptional regulation by P-Jun homodimers *in vivo*

Multiple lines of evidence suggest that Jun is able to homodimerize in the absence of Fos protein and bind to AP-1 binding sites (Halazonetis et al., 1988; Smeal et al., 1989). A caveat, however, to these experiments and similar experiments, is they are performed in cell culture, in cell free systems, or rely upon overexpression of Jun. Whether homodimerization of Jun at endogenous expression levels is able to activate gene expression remains unknown. In an attempt to extend tissue culture studies of Jun to animal models, I examined whether Jun-dependent transcription can occur in the absence of its partner, Fos.

To test whether excess Jun activity can compensate for the loss of Fos (consistent with the idea that in some contexts Jun homodimers might be bioactive), I examined the cuticle phenotypes of $puc^{1}kay^{1}$ double mutant embryos. *kayak* (*kay*) encodes the Drosophila homologue of Fos, and dimerizes with Jun to form the active AP-1 transcription factor that drives *dpp* transcription in the epidermis (Zeitlinger et al., 1997). In the Drosophila dorsal epidermis during dorsal closure, *kay* null mutants do not have active Jun-dependent transcription, as evidenced by a failure to transcribe leading-edge *dpp* (Zeitlinger et al., 1997). *puckered* (*puc*) encodes a Jun phosphatase, and mutants have increased bioactive P-Jun in the epidermis (Martín-Blanco et al., 1998). Thus, $puc^{1}kay^{1}$ embryos have an abundance of P-Jun due to loss of the Puc phosphatase, as well as an absence of AP-1, due to lack of Fos. $puc^{1}kay^{1}$ mutant embryos were collected and aged, and larval cuticle phenotypes were visualized after mounting in one-step mounting medium (30% CMCP-10, 13% lactic acid, 57% glacial acetic acid). $puc^{1}kay^{1}$ cuticles are indistinguishable from puc^{1} cuticles (Figure 5.1); as the *puc* mutant cuticle phenotype

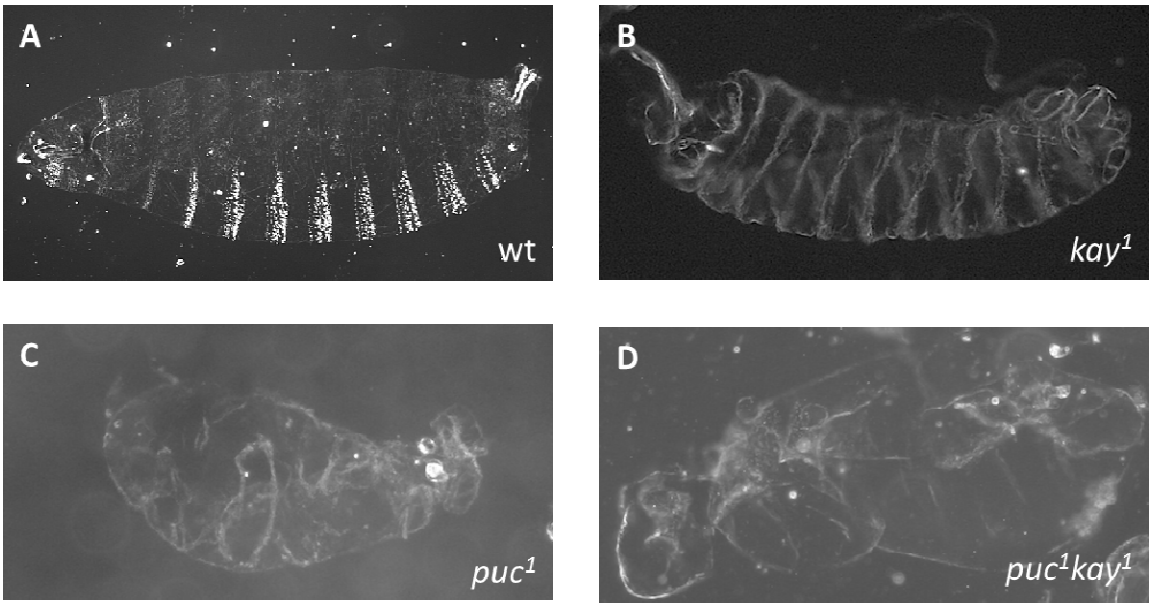

Figure 5.1 Jun homodimers transcribe *dpp in vivo.* A) A cuticle from a wild-type larva. B) *kay*^{1} cuticles are dorsal open and denticled on their ventral surface due to absences of Jun and Dpp activity. C) *puc*^{1} cuticles are dorsally puckered with hypotrophy of ventral denticle belts due to overactive Jun signaling and ectopic Dpp signaling. D) *puc*^{1}*kay*^{1} double mutant embryos secrete a cuticle resembling that of *puc*^{1} embryos, suggesting that Jun is able activate Dpp signaling ectopically even in the absence of its partner, *kay*-encoded Fos.

requires epidermal Dpp transcription, these data suggest that *dpp* is still transcribed in the epidermis of *puc*^{1}*kay*^{1} embryos. That the *puc kay* phenotype is not analogous to the *kay* phenotype suggests that the phenotype can arise from P-Jun acting on its own to enact transcription in the absence of Fos. Moreover, these data support a model wherein P-Jun can homodimerize and drive transcription *in vivo*, but only under conditions in which Jun phosphorylation levels are elevated. The next step in this evaluation of function of Jun homodimers in transcriptional activation is to examine the localization of *dpp* transcription in *puc*^{1}*kay*^{1} embryos.

Tests of Dpp autoregulation using the *dpp*^{151H} enhancer trap

Dpp expression is autoregulatory in various tissues, including imaginal disks and the midgut (Chanut and Heberlein, 1997; Yu et al., 1996). The disk region of the *dpp* gene lies in the 3' sequence and contains a series of cis-regulatory elements that control autoregulatory

expression of the *dpp* transcript (St Johnston et al., 1990). The disk region enhancer trap dpp^{151H} is reported to detect autoregulatory *dpp* expression in the Drosophila embryo through *lacZ* expression (Johnson et al., 2003; Smith et al., 1993). *mmy* and members of the CS synthesis pathway plays a role in antagonizing Dpp signal transduction and autoregulation, and thus we suspected that the dpp^{151H} enhancer trap might be a useful reagent for measuring signaling capacity in the epidermis of CS pathway mutants (Humphreys et al., 2013; Chapter 3).

To test the validity of this approach, I probed the expression of the enhancer trap dpp^{151H} in wt, mmy^{1}, and raw^{JG} backgrounds. Expression of the dpp^{151H} enhancer trap was visualized utilizing alkaline phosphatase immunolocalization methods; mouse anti-β-Gal (1:500, Promega) and goat anti-mouse alkaline phosphatase (1:2000, Promega) were utilized, as described (Sullivan et al., 2000). In wt embryos, β-Gal was expressed in the leading edge of the epidermis, as previously published (Figure 5.2A) (Johnson et al., 2003). Curiously, however, I saw no expansion of dpp^{151H} enhancer trap in the dorsolateral epidermis of mmy^{1} mutants (with hyperactive Dpp-dependent *dpp* transcription) (Figure 5.2B). Moreover, in *raw* embryos (with hyperactive Jun-dependent *dpp* transcription) a striking expansion of β-Gal expression in the dorsolateral epidermis was clear (Figure 5.2C). Together these data indicate that dpp^{151H} functions as a Jun reporter in the epidermis, rather than as a Dpp reporter.

To test the possibility that dpp^{H151} is a reporter of Jun signaling activity, dpp^{H151} expression should be assayed in *UAS-dpp* transgenic animals in which *dpp* expression is driven by the embryonic epidermal driver *69B-Gal4* (Brand and Perrimon, 1993); this analysis should be performed in wild-type and *Jra* mutant backgrounds (*Jra* encodes Drosophila Jun). If dpp^{151H} is a reporter of Dpp autoregulation, the reporter will turn on in response to *UAS-dpp* expression in the dorsal epidermis whether or not Jun is present. If dpp^{151H} is a Jun reporter, the reporter will still turn on in a *punt* or *thickveins* mutant background (where the Dpp signal cannot be transduced, and thus autoregulation could not occur) (Brummel et al., 1994; Letsou et al., 1995); this would indicate that it is not a reporter for autoregulatory Dpp, but is more likely a reporter for Jun signaling activity.

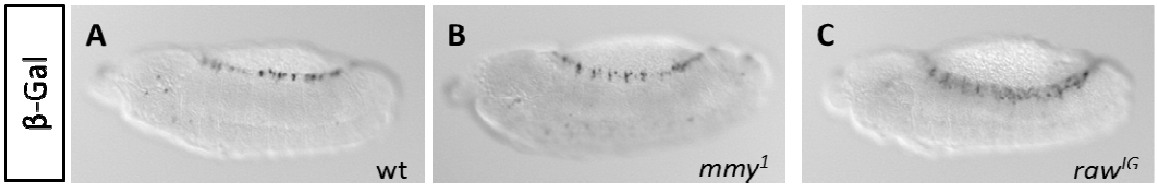

Figure 5.2. *dpp*^{151H} expression is restricted to the leading edge in *mmy* mutants. (A) Expression of the *dpp*^{151H} enhancer trap in wt embryos. In this background, expression is restricted to the leading edge of the epidermis. (B) In *mmy*^{1} mutants, the wt expression pattern of *dpp*^{151H} is replicated. (C) *raw*^{JG} mutants exhibit expanded *dpp*^{151H} expression in the dorsolateral epidermis.

The role of *super sex-combs* in Dpp signaling antagonism

In the RNAi screen for glycosyltransferases that antagonize Dpp signaling (described in Chapter 3), I identified *super sex-combs* (*sxc*), which encodes the Drosophila O-linked N-acetylglucosamine transferase, as a Dpp signaling antagonist. Sxc carries out reversible O-linked transfer of single GlcNAc onto serine/threonine residues in Drosophila (Sinclair et al., 2009).

Drosophila *sxc* is member of the Polycomb group, and the gene was originally characterized as a regulator of Hox gene expression (Gambetta et al., 2009; Ingham, 1984; Sinclair et al., 2009). *sxc* is recruited to Polycomb group targets on polytene chromosomes, and high levels of O-GlcNAc are also observed on chromosome-associated proteins in these same regions (Sinclair et al., 2009). It is of note that neither *sxc* recruitment nor O-GlcNAcylation is a prerequisite for assembly of Polycomb group proteins on chromosomal sites; assembly continues in the absence of *sxc* and O-GlcNAc (Sinclair et al., 2009).

To test for the role of Sxc in Dpp antagonism, early, ubiquitous *sxc* shRNA was driven under the control of Tubulin:Gal4 (described in Chapter 3). The UAS:shRNA line utilized was line v18610 from the Vienna Drosophila RNAi collection (Dietzl et al., 2007). Cuticles were analyzed for phenotypes suggesting a role in Dpp antagonism. I noted that the larval cuticle phenotype of *sxc* shRNA embryos is similar to that of the *raw* group Dpp signaling antagonists (including *mmy)*, in that there is a dorsal pucker, germband retraction defect, and hypotrophy of the ventral denticle belts (Figures 5.3A and 5.3B) (Bates et al., 2008; Byars et al., 1999; Humphreys et al., 2013).

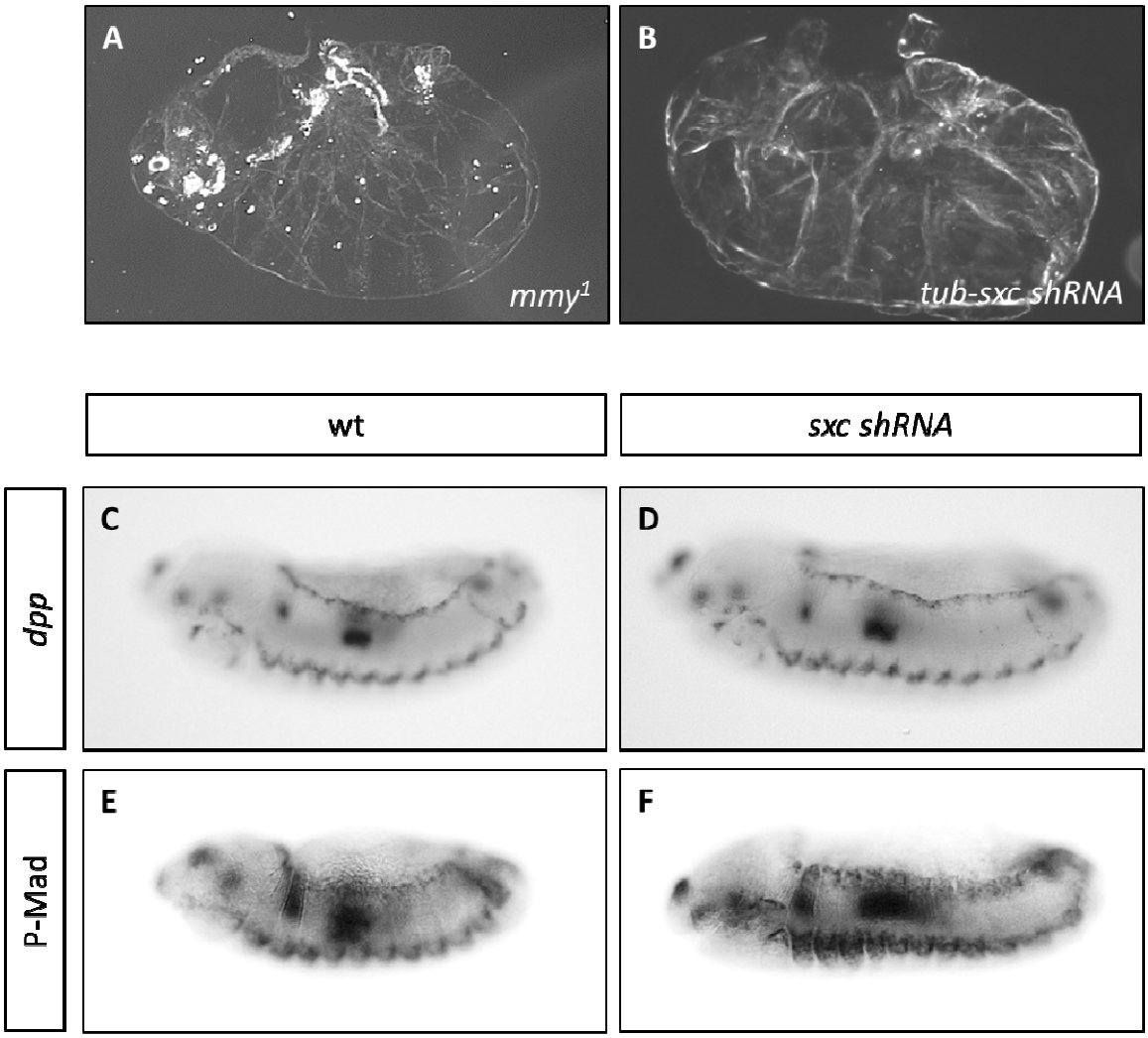

Figure 5.3. *sxc* loss-of-function cuticle phenotype results from ectopic Dpp signaling. (A) *mmy*^{1} mutant cuticles have a cuticle characterized by a dorsal pucker, a germband retraction defect, and hypotrophy of the ventral denticles. (B) *sxc* RNAi in embryos results in a *mmy*-like loss-of-function phenotype. (C) *wt* and (D) *sxc* RNAi embryos have restricted *dpp* transcription in the epidermis, unlike the expansion seen in *mmy*^{1} embryos (see Figure 3.1K). (E) Dpp signaling activity, measured by P-Mad, is found near the leading edge in wt embyros, (F) but is expanded in the dorsolateral epidermis in *sxc RNAi* embryos. This suggests the *sxc* RNAi cuticle phenotype is due to an increase in Dpp signaling, and not *dpp* transcription.

This cuticle phenotype suggested that *sxc* RNAi embryos might have an increase in dorsolateral epidermal Dpp signaling (Humphreys et al., 2013). To test this I probed Mad phosphorylation, as P-Mad is the transducer of Dpp signaling (Hoodless et al., 1996). I noted that, while epidermal Dpp signaling is mostly abolished in stage 13 wt embyros, *sxc* RNAi embryos have Dpp signaling expanded beyond the leading edge into the dorsolateral epidermis, not dissimilar to what we observe from *raw* and *mmy* mutants (see Figures 2.4F and 3.1K) (Byars et al., 1999). Next, I tested if this cuticle phenotype was associated with ectopic *dpp* transcription by probing *dpp* mRNA in stage 13 embryos. Unexpectedly, *dpp* mRNA remains restricted to the leading edge of the epidermis, whereas previously I have only observed epidermal Dpp signaling expansion in the presence of ectopic *dpp* (Humphreys et al., 2013). These data suggest that *sxc* loss of function results in Dpp signaling expanding from the transcriptional source, but its level is not sufficient to activate all its transcriptional targets (e.g., *dpp*).

It is highly likely that *mmy* loss of function would have an impact on intracellular O-GlcNAc levels. There is some evidence that when UDP-GlcNAc levels are depleted, intracellular O-GlcNAc levels will be more sensitive to a reduction than other glycosylation processes. When UDP-GlcNAc was disrupted in MEF cell culture by EMeg32 knockout, cytoplasmic and nuclear O-GlcNAc levels were more affected than other glycosylmodifications (Boehmelt et al., 2000). Supplementing the cells with additional GlcNAc via culture medium led to restoration of wild-type cytoplasmic O-GlcNAc levels and elimination of growth defects (Boehmelt et al., 2000). The case of *sxc* modulation of Dpp signaling sensitivity is intriguing because it is biochemically downstream of *mmy*, and yet does not have *dpp* expansion. The O-GlcNAcylated target must be intracellular (as Sxc only modifies intracellular targets) and must be no further downstream in the signaling pathway than Mad itself (as the phenotype is manifest at this point). This leaves very few well-characterized potential targets, being Mad, Tkv, and Punt. As O-GlcNAcylation can be mutually exclusive to phosphorylation (Comer and Hart, 2001), O-GlcNAc may be another method present in the Drosophila embryo to prevent small amounts of Dpp from enacting a strong Dpp signaling response. Further research characterizing the signaling components that are specifically modified in order to modulate sensitivity of Dpp signaling machinery to the Dpp ligand is ongoing

in the lab.

Characterization of *CG33181*, a potential Dpp antagonist

In 2002, Alain Vincent's lab group published the results of an X-chromosome P-insertion mutagenesis screen isolating and localizing mutants that are homozygous lethal (Bourbon et al., 2002). One of the mutants generated in this screen, $CG33181^{PL112}$, had a cuticle phenotype with hypotrophy of the ventral denticles. As hypotrophy of the ventral denticle belts is a phenotypic characteristic of members of the raw group on JNK and/or Dpp signaling antagonists, I obtained this mutant line from the investigators to characterize its effects on JNK and Dpp antagonism. In cuticle preparations, I noted that there was a fully penetrant hypotrophy of the ventral denticles, though some denticles still remained; thus, the phenotype is similar to what we observe in several *mmy* mutant alleles, including mmy^{LM16} embryos (Figures 5.4A and 5.4B). Additionally, what the investigators identified as "melanotic patches found in salivary glands" are similar to stable salivary gland structures that form in many different *mmy* mutant background, including mmy^{LM16} embryos. As the hypotrophy of ventral denticles is a hallmark of ectopic *dpp* in the epidermis (Bates et al., 2008; Byars et al., 1999; Humphreys et al., 2013), I obtained the $CG33181^{PL112}$ mutant line in order to characterize its *dpp*-associated phenotypes.

$CG33181^{PL112}$ mutants maintain normally restricted *dpp* transcription in the epidermal leading edge and are indistinguishable from wild-type embryos (Figures 5.4C and 5.4D). However, two points are worth consideration to understand this phenotype. First, multiple *mmy* alleles that have similar cuticle phenotypes to $CG33181^{PL112}$ mutants do not have ectopic *dpp* transcription (data not shown). Second, the cuticle phenotype of denticle hypotrophy can result from increased Dpp signaling, even if *dpp* transcription remains unaffected. This conclusion is supported by my experiments on loss of function of *sxc* (Sinclair et al., 2009); embryonic *sxc* RNAi results in expanded Mad phosphorylation in the dorsolateral epidermis, even though *dpp* transcription remains restricted to the leading edge of the epidermis (Figures 5.3D and 5.3F). Research into the mechanism of the expansion of P-Mad in *sxc* loss-of-function embryos is ongoing, but it does demonstrate that Dpp signaling activity can be ectopically expanded even

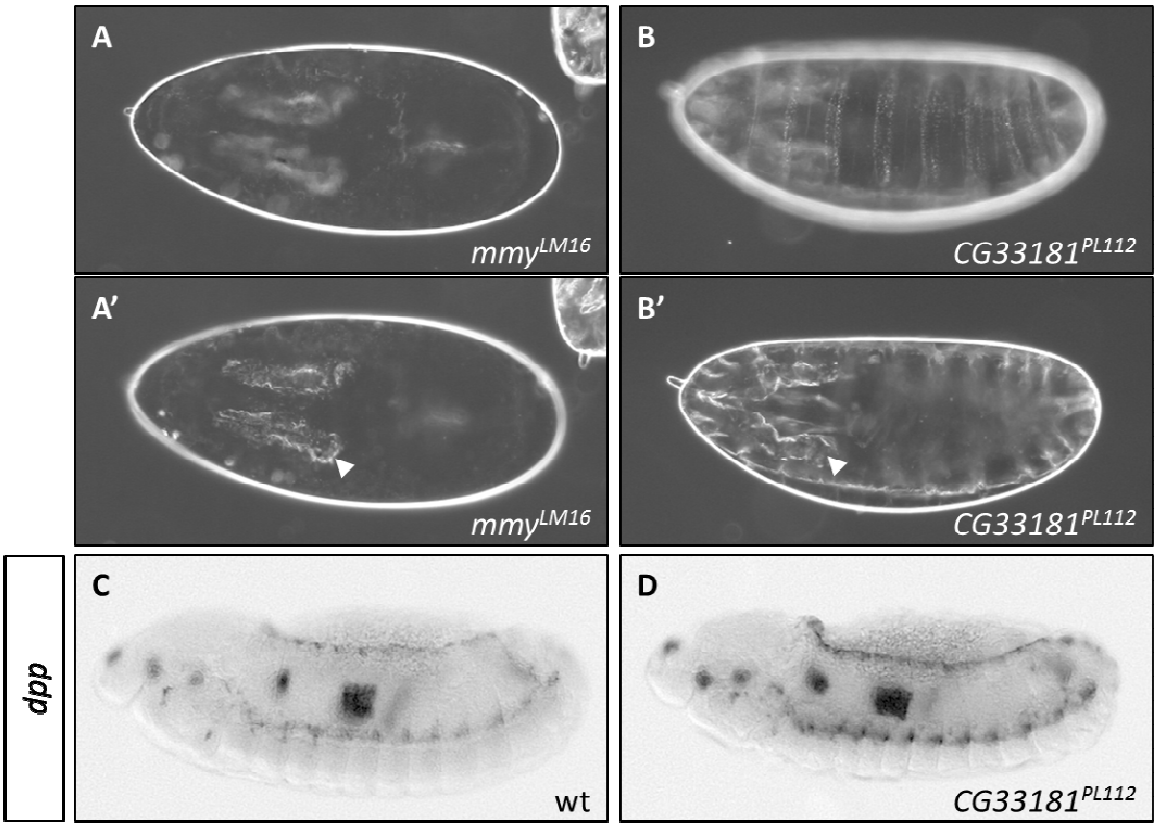

Figure 5.4. Phenotypic characteristics of *CG33181*^{PL112} mutant embryos. (A) *mmy*^{LM16} cuticles exhibit hypotrophy of ventral denticle belts. Also of note is the acid-resistant salivary gland remnant (A') denoted with a white arrow. (B) *CG33181*^{PL112} mutant cuticles similarly exhibit hypotrophy of ventral denticle belts and (B') acid-resistant salivary gland remnants. (C) Wild-type and (D) *CG33181*^{PL112} mutant embryos have *dpp* restricted to the leading edge, indicating that CG33181 does not antagonize *dpp* transcription. All cuticles are shown in ventral views, and embryos are shown in lateral views.

though *dpp* transcription itself appears unaffected. Mad phosphorylation should be examined in *CG33181*^{PL112} mutant embryos to determine if it has expanded Dpp signaling activity, in a similar manner.

The protein encoded by *CG33181* is homologous to human SLC41A2 (Solute carrier family 41, member 2), a membrane-embedded divalent cation transporter. This protein can transfer Mg2+ and Mn2+ across cell plasma membranes (Sahni et al., 2007). This is not the first ionic channel with potential effects on Dpp signaling; Dpp signaling in Drosophila wing discs is reduced when the K+ channel Irk2 function is depleted (Dahal et al., 2012). It is intriguing to speculate that Mg modulates Dpp signaling thresholds by altering Mad function via activity of a

Drosophila homologue of Protein phosphatase magnesium-dependent 1A (PPM1A). PPM1A is a phosphatase which requires Mg2+ for activity (Duan et al., 2006; Lin et al., 2006). PPM1A antagonizes Mad via removal of phosphate groups, resulting in the inactivation of Mad and its exit from the nucleus. Thus, loss of cellular Mg2+ could result in loss of PPM1A activity and thus a higher level of Mad phosphorylation in response to wild-type levels of Dpp.

Tests for the role of *mmy* in Wingless signal regulation during embryonic development

The glypicans Dally and Dally-like regulate multiple signaling factors during Drosophila development, including Wingless (Wg) and Hedgehog (Hh) (Akiyama et al., 2008; Desbordes and Sanson, 2003; Gallet et al., 2008; Lin and Perrimon, 1999; Wu et al., 2010; Yan et al., 2009). As *mmy* is required for synthesis of the heparan sulfate attachments on glypicans, I hypothesized that *mmy* loss of function may have an effect on embryonic Wg and Hh signaling. To test for disruptions in epidermal Wg, which helps define segment polarity, I examined embryonic localization of Engrailed and Even-skipped in whole mount embryo immunolocalization studies (4D9 and 2B8, DSHB). Wg and Hh signaling contribute to the establishment and maintenance of En expression in organized segmental epidermal stripes (Figure 5.5A). Embryonic En pattern was compared among wild-type, *wg,* and two *mmy* mutant alleles, mmy^{LM24} and mmy^{LM47} embryos. In *wg* mutants, there is a decay of En expression in the epidermis, with the only remaining protein seen in the extreme ventral ectoderm (Figure 5.5B). There was no difference between the En pattern observed between wild-type embryos and *mmy* mutants (Figures 5.5A, 5.5C, and 5.5D). These data suggest that *mmy* does not play a role in Wg pattern regulation in the ventral epidermis. I examined Eve expression as well to determine if *mmy* was required for Wg signaling in the dorsal epidermis; definition of the Eve cells of the dorsal mesoderm requires proper Wg and Hh patterning for correct specification (Figures 5.5E and 5.5F) (Wu et al., 1995). The loss or gain of Eve-expressing pericardial cells in the dorsal mesoderm would indicate that *mmy* is required for Wg regulation dorsally; this region is near the area where *mmy* regulates Dpp signaling in the dorsal epidermis (Humphreys et al., 2013). I observed no loss, gain, or

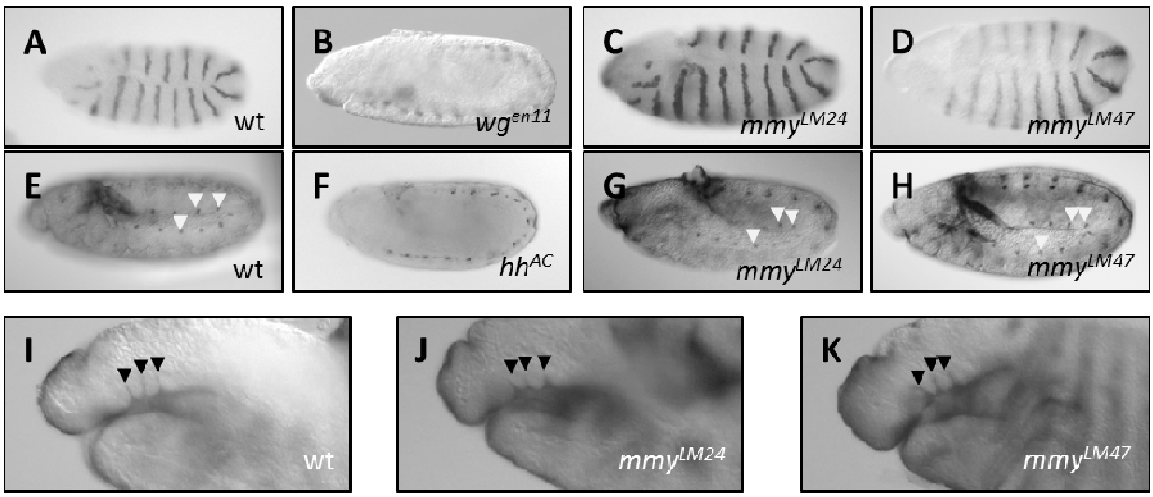

Figure 5.5. *mmy* loss of function does not affect embryonic Wg and Hh signaling. (A) Engrailed (En) is expressed in segmental epidermal stripes in wt embryos. (B) *wg* mutants lose most of the epidermal En expression. (C) There are no En expression abnormalities in (C) mmy^{LM24} and (D) mmy^{LM47} mutants, suggesting *mmy* loss of function does not affect Wg signaling in the epidermis. (E) Proper Wg and Hh signaling are required to properly specify the Eve-expressing pericardial cells of the ventral mesoderm (white arrows) in wt embryos. (F) hh^{AC} mutants have disrupted Wg signaling in the mesoderm; as a result, the Eve-expressing pericardial cells are not specified. (G) Eve-expressing pericardial cells are properly specified in the mesoderm of (C) mmy^{LM24} and (D) mmy^{LM47} mutants, suggesting *mmy* loss of function does not affect Wg signaling in the mesoderm. (I) Wg signaling is required for the formation of three distinct invaginations (black arrows) of the primordial stomatogastric nervous system (González-Gaitán and Jäckle, 1995). These structures form properly in (C) mmy^{LM24} and (D) mmy^{LM47} mutants, indicating *mmy* is not required to establish or maintain proper Wg signaling in this region.

mispatterning of Eve-expressing pericardial cells in the dorsal epidermis in *mmy* mutants, suggesting that mmy does not regulate Wg signaling in the dorsal epidermis.

To test for disruptions in Wg in a polarity-independent function, I examined the development of the stomatogastric nervous system (SNS) by visualizing it with anti-Crumbs antibody (Cq4, DSHB) (Figure 5.5I). SNS development is Wg-dependent but independent of segment polarity (Häcker et al., 1997). I observed no defects in the SNS in any of the *mmy* mutant lines tested (Figures 5.5J and 5.5K), indicating *mmy* loss does not influence Wg signaling in SNS development.

The lack of Wg-associated phenotypes in *mmy* mutants is surprising. It is known that the glypicans Dally and Dally-like are required for proper Wg and Hh patterning in the ventral epidermis (Desbordes and Sanson, 2003; Nakato et al., 1995). Loss of maternal and zygotic

Dally or Dally-like results in cuticle defects due to aberrations in segment polarity. It is also known from my analysis of Dally-like by western blot (see Chapter 3) that *mmy* mutants have a decrease in both HS synthesis and a decrease in Dally-like protein. These data suggest that, though there is a decrease in both HS and Dlp in *mmy* mutants, there is not a sufficient decrease in order to significantly affect Wg and Hh signaling. Even a null allele of *dlp* does not have a *wg*-like mutant phenotype, but only exhibit *wg*-like cuticle phenotypes when depleted by RNAi (Desbordes and Sanson, 2003). Maternal and zygotic loss of function of *mmy* may be required to elicit an effect on Wg and/or Hh signaling. Even the reduced level of UDP-GlcNAc in *mmy* mutants is sufficient for embryonic Wg and Hh signaling.

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